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Effect of redox potential on rat liver F_1-ATPase

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Redox titration of F_1-ATPase from rat liver mitochondria referred to the modification of the hydrolytic activity on Mg-ATP has resulted in a three-step pattern, with three distinct jumps of activity separated by clear plateaus. The measured potentials ranged from −400 mV to +400 mV and were obtained by the addition of dithionite and ferricyanide. Electron exchange was facilitated with a mixture of different redox mediators. At pH 7.4 the midpoint potentials were +210 mV, +40 mV and −230 mV. These three midpoint potentials were displaced towards more negative values by 2,4-dinitrophenol or by an increase of the pH of the medium. The titration curves were described by $n=2$ Nernst equations.

Mitochondrial ATPase; F_1-ATPase; Redox titration

1. INTRODUCTION

A great variety of compounds of different nature can influence the hydrolytic activity of mitochondrial ATPase. One of these compounds is the reducing agent dithionite which has been shown to increase the hydrolytic ATPase activity of mitochondria [1] as well as that of the isolated and purified enzyme [2]. The effect on the isolated enzyme could be reverted by the oxidizing agent dichlorophenolindophenol, an observation which was tentatively interpreted as a reversible modification of F_1-ATPase by a redox reaction [2]. Metabolic situations leading to an increase in the reduction power within the mitochondria, as reflected by a rise of [NADH]/[NAD^+] and [Total flavin]/[FAD] ratios were accompanied by an increase in ATPase hydrolytic activity [3]; this effect was also ascribed to a redox dependent change.

These observations prompted us to carry out the titration of the possible redox centre or centres in the enzyme. This paper reports the results obtained, which suggest the existence of three midpoint potentials of F_1-ATPase detected through the changes in enzyme activity upon varying the redox potential of the medium.

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2. MATERIALS AND METHODS

Rat liver mitochondria were isolated by the method of Hogeboom [4]. Protein determination was carried out following the technique of Lowry et al. [5]. F_1-ATPase was prepared from rat liver mitochondria by the procedure of Lambeth and Lardy [6].

Redox potentiometry was carried out essentially by the procedure of Dutton [7] in an anaerobic cuvette, continuously flushed with oxygen-free nitrogen, magnetically stirred and immersed in a water bath at 12°C, a temperature which is adequate to maintain the enzyme unaltered. The oxidation reduction potential was measured using a platinum electrode (Radiometer Type P101) in combination with a standard calomel electrode (Radiometer Type K401) and a potentiometer (Model Keithley 642). Enzyme concentration was approximately 0.06 mg/ml. Redox mediation between F_1-ATPase and the electrodes was provided by benzylviologen ($E_{m7.0} = -311$ mV), phenosafranine ($E_{m7.0} = -239$ mV), 2-OH-naphthoquinone ($E_{m7.0} = -145$ mV), duroquinone ($E_{m7.0} = +7$ mV), phenazinemethosulphate ($E_{m7.0} = +80$ mV), dichlorophenolindophenol ($E_{m7.0} = +217$ mV) and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine ($E_{m7.0} = +260$ mV). The buffer system used was 50 mM Tris-SO_4, 1 mM EDTA and 2 mM ATP, pH 7.4.

The potential of the reaction mixture was adjusted using freshly prepared solutions of either potassium ferricyanide or sodium dithionite. Oxidative titrations were performed by adding 5–10 μl aliquots from adequate solutions, ranging in concentrations from 2 to 50 mM, with a microsyringe through a septum in a sidearm of the cuvette. Samples of 100 μl were

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transferred to pre-gassed tubes and ATPase activity determined under a continuous nitrogen current. The samples were preincubated for 5 min at 30°C and the reaction initiated by the addition of Mg-ATP dissolved in Tris buffer, pH 7.4. The final concentration of substrate was 7.5 mM and that of the buffer 50 mM. The incubation was continued for 1 min and stopped by the addition of trichloroacetic acid. Inorganic phosphorus was measured according to Fiske and Subbarow [8].

Controls were treated in the same way as the samples being titrated, except that buffer solution was added instead of that of the oxidative or reducing agent. No change in ATPase activity, ascribable to the manipulations of the method, was observed.

3. RESULTS AND DISCUSSION

The most widely used technique for the measurement of midpoint potentials (E_m) in biological systems is redox potentiometry. This procedure requires that some property of the biological molecule under consideration be monitored as a function of the ambient redox potential [7]. In the present work the parameter explored was the hydrolytic activity of the enzyme.

Fig.1 shows the Nernst plot for the oxidative titrations of rat liver F_1-ATPase. Both dots and circles correspond to experimental data, whereas solid curves are theoretical lines for two-electron accepting centres, which provide a good visual fit to the data.

When setting up the experimental conditions under which redox determinations were to be carried out, the possible influence of the mediators on the enzyme activity was also tested. No effect on the activity was observed when determined in the presence of the mixture of mediators at the concentrations ranging from half up to four times those shown in fig.1 and finally chosen for the titration experiments.

In some experiments redox titrations were carried out in the presence of the mixture of mediators at concentrations 1.5 times those indicated in fig.1 and the E_m values remained unaffected.

The titrations suggest the existence of centres with different midpoint potentials in the protein. The values of the $E_{m7.4}$ of the different centres were $E^1_m = +210$ mV, $E^2_m = +40$ mV and $E^3_m = -230$ mV with an accuracy of about $\pm$ 10 mV.

Fig.1 also shows the midpoint potentials of F_1-ATPase in the presence of 2,4-dinitrophenol. The titration curve was significantly affected causing a displacement of approximately 100 mV towards the more negative values. The new $E_{m7.4}$ values were $E^1_m = +110$ mV, $E^2_m = -45$ mV and $E^3_m = -320$ mV. The E_m shifts induced by dinitrophenol could be the expression of conformational changes caused by the interaction of this compound with the protein. The modification of the E_m values could be related to its uncoupling effect.

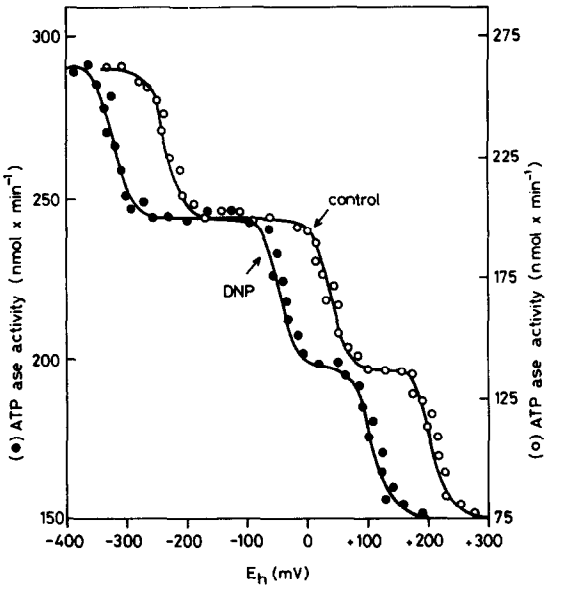


Fig.1. Redox titration of rat liver purified F_1-ATPase. Oxidation-reduction mediators added for the high potential region were 30 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine and 40 μM dichlorophenolindophenol; for the intermediate potential region the mediators added were 40 μM dichlorophenolindophenol, 63 μM phenazinemethosulphate and 25 μM duroquinone. And for the low potential region the mediators were 100 μM 2-hydroxynaphthoquinone, 100 μM phenosafranine and 200 μM benzylviologen. The activity has been referred to 6 μg of purified enzyme protein. When present the concentration of DNP was 2 mM.

Titrations carried out at pH values higher than those of the control led to a displacement of midpoint potentials towards more positive values. At pH 7.8 these values were as follows: $E^1_m = +90$ mV, $E^2_m = -90$ and $E^3_m = -340$ mV, as calculated from the curve in fig.2. At pH 8 $E^1_m = +50$ and $E^2_m = -130$ (titration curve not shown). At this latter pH, it proved difficult to obtain redox potentials sufficiently low and stable to guarantee an accurate determination of the expected plateau of ATPase activity at those negative

values. However, the shape of the titration curves suggests an E_m^3 approximately equal to -410 mV.

The pH dependence of E_m (approx. -300 mV/pH unit) would mean the coupling of 10 protons per redox reaction ($n = 2$). Such a high ratio could be explained by pK changes of acid/base groups in the enzyme as a consequence of possible conformational changes induced by redox reactions. In all cases titration curves were described by $n = 2$ Nernst equations.

With the purpose of determining if the Nernst curves obtained reflected the existence of redox centres in purified F_1-ATPase, a stoichiometric titration of the enzyme was carried out in the complete absence of redox mediators. Dithionite was used as reductant. To 4 ml of a 0.5 mM sodium dithionite solution, freshly prepared, and kept under nitrogen, with a redox potential of -270 mV, 0.08 μmol of F_1-ATPase (assuming an M_r of 360000) dissolved in 2 ml of 50 mM Tris buffer, 2 mM ATP, pH 7.8 were added provoking a fast increase of potential of 420 mV. It was verified that the addition of buffer, prepared in the same way and continuously flushed with oxygen free nitrogen, as in the case of F_1-ATPase, did not cause any appreciable change in the potential, even after a period as long as 40 min, when added to an aliquot of the same dithionite solution. It was equally found that 0.16 μmol of albumin dissolved in 4 ml added to another aliquot of the dithionite solution did in no way affect the initial potential. To calculate the approximate number of electrons donated by dithionite, its solution was titrated with dichlorophenolindophenol. To reach a rise of 420 mV over the initial redox potential of a 4 ml volume of a freshly prepared 0.5 mM sodium dithionite, 0.238 μmol of the oxidant were required. These data suggest the acceptance of approximately six electrons by each F_1-ATPase complex.

The fact that a property of the enzyme such as its hydrolytic activity was affected by the redox potential of the medium, and the fact that F_1-ATPase itself accepted electrons, may be the expression that the enzyme itself, or ligands bound to it, undergo some redox reaction. The coincidence of the hydrolytic activity with the Nernst equation, and the existence of three midpoint potentials with values approximately placed within the jumps of potential of the mitochondrial electron transfer

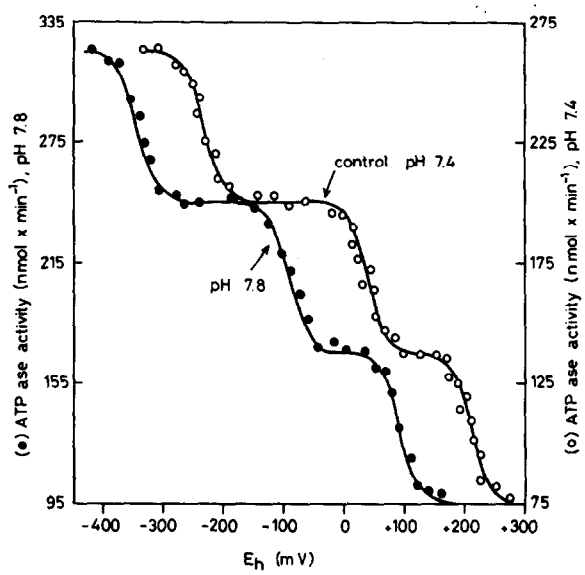


Fig.2. Redox titration of F_1-ATPase at pH values of 7.4 and 7.8. The rest of the experimental conditions were the same as those indicated in fig.1.

chain are quite suggestive. Although the precise meaning of this observation is, for the moment, far from being clear, they pose questions such as the possible direct coupling of oxidation and phosphorylation, or the coupling of the hydrolytic reaction to a reverse electron flow by the enzyme itself, or they might simply be the expression of some regulatory mechanism of the hydrolysis of ATP, or of its synthesis [2]. These observations could fit into a rather recent scheme proposed by Slater et al. [9] according to which the interactions between ATPase and the oxidized or reduced forms of the electron carriers could be the basis for the energy transducing mechanism. The data now presented seem to favour the participation of closely associated redox centres in at least some of the catalytic processes carried out by the enzyme.

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