AN ESSENTIAL ARGINYL RESIDUE IN THE SOLUBLE CHLOROPLAST ATPase

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1. Introduction

The photosynthetic formation of ATP in chloroplasts requires a coupling factor with latent ATPase activity, which has been purified to homogeneity [1,2], has a molecular weight of 325 000 [1] and is made up of five different subunits [3].

Studies intended to characterize the active site(s) of the coupling factor have been carried out by chemical modification with *N*-ethylmaleimide [4,5], 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole [6], *o*-iodosobenzoate [7], 2,2′-dithio-bis-(5-nitropyridine) [8,9] and trypsin treatment [6]. Inhibition of the ATPase activity of the chloroplast coupling factor by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole suggests the involvement of tyrosine residues in the catalytic site [6].

The participation of arginyl residues in the binding of anionic substrates or ligands in several enzymes have been shown by chemical modification with α-dicarbonyl reagents [10–14]. Recently, Borders et al. [10] suggested that the adenine nucleotide binding site of creatine kinase contains an essential arginyl residue and Marcus et al. [11] found that modification of an arginyl residue of the mitochondrial ATPase, probably at the hydrolytic site, resulted in enzyme inactivation.

The present paper reports the effects of an arginine-specific reagent, 2,3-butanedione in borate buffer, on the ATPase activity of the purified spinach chloroplast coupling factor. The results obtained suggest that there is one essential arginine per active site in the chloroplast ATPase.

2. Experimental

Spinach coupling factor 1 was prepared and purified according to Lien and Racker [15]. The step of sucrose density-gradient centrifugation was omitted. Preparations with a specific Ca^{2+}–ATPase activity after trypsin activation of about 18 μmol P_i. min^{-1}. mg^{-1} were used. Polyacrylamide gel electrophoresis showed the coupling factor 1 to be at least 95% pure.

Aliquots of enzyme, stored at 4°C in 2 M $(NH_4)_2SO_4$, were centrifuged for 10 min at 10 000 × *g* and appropiately diluted with 50 mM boric acid–borax buffer (pH 7.8). The trypsin activation of latent Ca^{2+}-ATPase of coupling factor 1 was performed as described [15] except that the reaction medium was 50 mM borate buffer (pH 7.8).

ATPase activity was measured at 37°C in final vol. 1 ml containing 40 mM Tris–HCl (pH 8), 5 mM ATP, and 5 mM $CaCl_2$. The reaction was started by addition of 3–5 μg of trypsin-activated coupling factor. After 10 min, the reaction was stopped by addition of 0.1 ml trichloroacetic acid 50% (w/v). In the supernatants the P_i liberated was determined colorimetrically according to Sumner [16].

Protein was determined according to Lowry et al. [17], with bovine serum albumin as the standard.

Modification of ATPase activity of coupling factor 1 with 2,3-butanedione was carried out at 25°C in a reaction medium (0.5 ml) containing 50 mM borate buffer (pH 7.8), appropiate concentration of 2,3-butanedione and 100 μg trypsin-activated ATPase. Solutions of 2,3-butanedione were

freshly prepared for each experiment. Concentrations 10-fold higher than required for modification were prepared in 50 mM borate buffer (pH 7.8); the pH of solutions were readjusted with 1 N NaOH. No further adjustments of pH were required during the incubation time.

Trypsin, trypsin inhibitor, ADP and ATP were obtained from Sigma Chemical Co (USA). 2,3-Butanedione was obtained from BDH Chemicals Ltd (England).

All other chemicals were of analytical grade.

3. Results and discussion

Figure 1 shows that incubation for 120 min of the purified chloroplast coupling factor with increasing concentrations of 2,3-butanedione in a borate buffer resulted in inhibition of the trypsin-activated Ca^{2+}-ATPase activity of the enzyme. Complete inhibition was achieved with longer incubation times. Similar treatment of the native coupling factor prior to activation of the latent ATPase by either trypsin or heat resulted also in complete inhibition (not shown).

The semilogarithmic plot of fig.2 shows that the inactivation rates follow apparent first-order kinetics until the loss of activity exceeds about 85%. A slope of 0.84 was obtained when these data were plotted as the log $(1/t_{0.5})$ against the log of butanedione concentration as shown in fig.2B. The slope gives the number

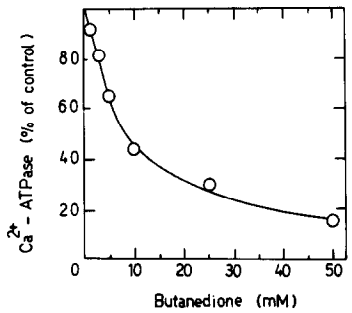

Fig.1. Inhibition of Ca^{2+}-ATPase activity of chloroplast coupling factor 1 by 2,3-butanedione. Trypsin-activated ATPase was modified by preincubation with the indicated concentrations of 2,3-butanedione during 120 min as described in the text. After this period the ATPase activity was determined as described. Control value was 18.9 μmol P_i. min^{-1}. mg^{-1}.

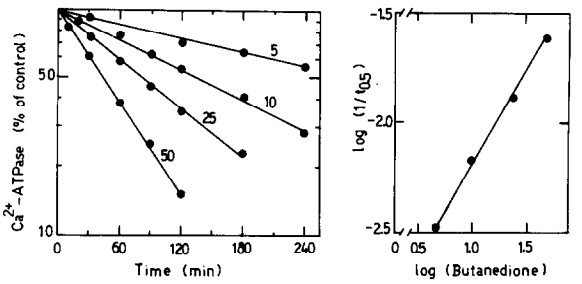

Fig.2. Time-dependence of the inactivation of Ca^{2+}-ATPase of chloroplast coupling factor 1 by 2,3-butanedione in borate buffer. Experimental conditions were as described in the text. (2A) Semilogarithmic plot of remaining Ca^{2+}-ATPase activity after treatment with the stated concentrations of butanedione (numerals on the slopes, mM). (2B) Apparent order of the reaction between ATPase and 2,3-butanedione with respect to 2,3-butanedione. Half-time of inactivation $(t_{0.5})$ values were calculated from the data of fig.2A and plotted as shown.

of molecules of inhibitor reacting per active site of the enzyme [11,18–21]. This suggests that the inhibition of the ATPase activity by butanedione–borate is the consecuence of blocking one arginine residue per active site of the enzyme.

As shown in fig.3 the inhibition of the ATPase

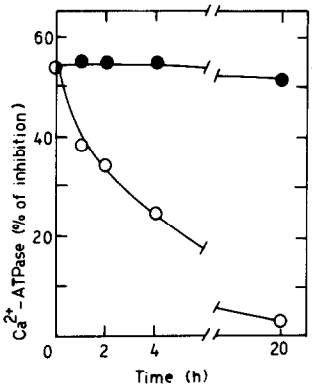

Fig.3. Reversibility of Ca^{2+}-ATPase inactivation on the removal of borate. Duplicate aliquots (200 μg) of ATPase were incubated with 50 mM 2,3-butanedione in borate buffer as described in the text. After 45 min each fraction was precipitated twice by addition of an equal volume of saturated $(NH_4)_2SO_4$. Then one fraction was resuspended in 50 mM borate buffer (pH 7.8) and the other in 50 mM Tris–HCl (pH 7.8). The ATPase activity of the enzyme resuspended in 50 mM Tris–HCl (pH 7.8) (o–o) or in 50 mM borate buffer (pH 7.8) (•–•) was determined at the times indicated.

activity by butanedione was completely reversible when, after an incubation of 45 min with 50 mM butanedione in borate buffer, the reagent was eliminated by ammonium sulphate precipitation of the enzyme followed by resuspension in a Tris–HCl buffer. If the precipitated enzyme was resuspended in borate buffer, the activity of the partially inhibited enzyme remained stable for at least 20 h and no reactivation was observed. The reversibility of butanedione inhibition of chloroplast ATPase in the absence of borate buffer is in agreement with the behaviour of other enzymes, including the mitochondrial ATPase, as described by other workers [10–14]. When the incubation of the ATPase with 25 mM 2,3-butanedione was carried out for 2 h substituting Tris–HCl buffer for the borate buffer, the inhibition was only 7% instead of 64%.

The inhibition of the ATPase by butanedione depended not only on the presence of borate buffer but it was also affected by the presence of the substrate. Thus, 25 mM ATP afforded a considerable protection of the ATPase activity of chloroplast coupling factor against inactivation by 25 mM butanedione in borate buffer since in its presence the $t_{0.5}$ was raised from 80–210 min. ADP (2 mM) also afforded some protection ($t_{0.5}$ = 130 min) while $MgCl_2$ was without effect.

The stability of the modified enzyme in borate buffer and the slow reversion of the inhibition by butanedione (fig.3) allowed us to study the kinetics of partially inhibited ATPase. Figure 4 shows that the modified enzyme had a lower V_{max} but the same K_m for ATP. The allosteric inhibition of the Ca^{2+}-ATPase activity of the chloroplast coupling factor by ADP [22] was not affected in the modified enzyme as shown in fig.5.

The chloroplast coupling factor has 180 arginyl residues [1] but the inhibition of its ATPase activity by butanedione–borate can be related to the modification of only one residue per active site according with our results. This does not exclude that other, non essential arginine(s) may be modified by the reagent.

The role of this essential arginyl residue in the active site of the ATPase can not be defined by the present results but it may be related to the binding of ATP, as suggested by the protection afforded by ATP. Similar suggestions have been put forward by other authors working with creatine kinase [10], mitochondrial ATPase [11], glutamine synthetase and carbamyl phosphate synthetase [14].

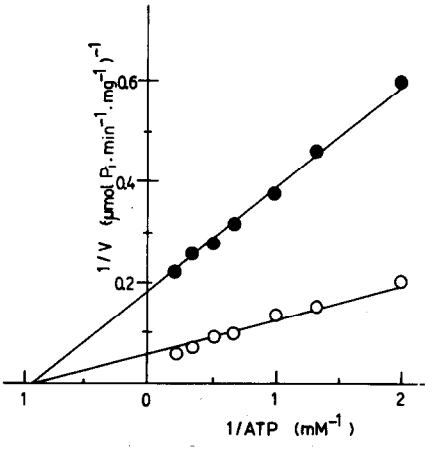

Fig.4. Double reciprocal plots for ATP hydrolysis by unmodified (o–o) and 2,3-butanedione modified (•–•) Ca^{2+}-ATPase of coupling factor. The incubation with 10 mM 2,3-butanedione during 180 min was performed as described in text.

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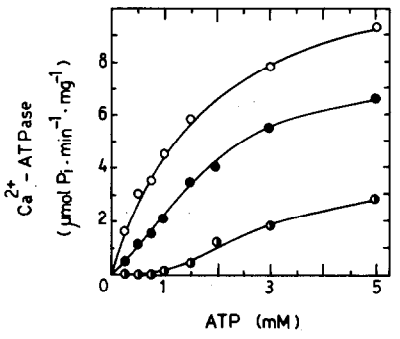

Fig.5. Allosteric inhibition by ADP of 2,3-butanedione modified Ca^{2+}-ATPase. Modification of 100 μg Ca^{2+}-ATPase with 50 mM 2,3-butanedione was performed during 60 min as described in text. After the modification period, the assays for ATPase activity were performed in the absence of ADP (o–o) or in the presence of 0.5 mM ADP (•–•) or 1 mM ADP (•–•).

coupling factor. This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina) and the Fundación Cherny (Argentina). The authors are Career Investigators of the former institution.

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