ESSENTIAL ROLE OF AN ARGINYL RESIDUE AT THE CATALYTIC SITE(S) OF CHLOROPLAST COUPLING FACTOR

Rubén H. VALLEJOS, Alejandro VIALE and Carlos S. ANDREO

Centro de Estudios Fotosintéticos y Bioquímicos, Consejo Nacional de Investigaciones Científicas y Técnicas – Fundación M. Lillo and *Universidad Nacional de Rosario, CEFOBI – Suipacha 531, 2000 Rosario, Argentina*

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1. Introduction

Spinach chloroplasts have a latent ATPase involved in the photosynthetic synthesis of ATP which is remarkably similar to the mitochondrial and bacterial ATPases associated also with the synthesis of ATP [1]. The chloroplast ATPase or coupling factor 1 is a complex protein made up of five different subunits and with mol. wt 325 000 [2,3]. It can easily be detached from the thylakoid membrane with the concomitant loss of photophosphorylation activity of the latter [4]. The soluble coupling factor has also a latent ATPase activity which may be activated by heating or trypsin treatment but this activation is associated with an irreversible loss of the coupling activity [5,6].

Chemical modification of soluble CF_1 has allowed a partial characterization of the catalytic site. Thus, Deters et al. [7] have found that there is an essential tyrosine residue in soluble CF_1 localized in the β subunit and we have recently found [8] that modification of one arginyl residue per catalytic site in purified CF_1 by 2–3 butanedione resulted in complete inhibition of its ATPase activity.

The demonstration [9–11] that phenylglyoxal and 2,3-butanedione in borate buffer are useful reagents for arginine residues of proteins under mild conditions has lead to the description of the functional role played by arginine residues in several enzymes [8,11–16].

One important function of arginine residues is their participation in the binding of anionic ligands to the proteins [11–14]. In particular they were identified at the NAD-binding sites of several enzymes [12]. Arginyl residues were also found at the ATP binding sites of creatine kinase [13], glutamine synthetase, carbamyl phosphate synthetase [14], mitochondrial ATPase [15] and chloroplast ATPase [8] and at the GTP binding site of *E. coli* elongation factor G [16].

The present paper shows that incubation of spinach chloroplasts with phenylglyoxal resulted in complete inhibition of its photophosphorylation, ATP–P_i exchange and Mg–ATPase activities. Photophosphorylation was less sensitive to the reagent than the other activities. Adenine nucleotides protected against inactivation. The Ca–ATPase and coupling activity of soluble CF_1 were similarly inhibited. Modification of only one arginine residue per active site in CF_1 was responsible of the effects described although the arginine involved in the sites of ATP synthesis and hydrolysis might be different.

2. Experimental

Chloroplasts were isolated from fresh market spinach leaves (*Spinacea olearacea* L) as described [17] except that leaves were homogenized in a blendor in 175 mM NaCl, 5 mM $MgCl_2$ and 25 mM tricine–NaOH (pH 8) and finally suspended in the same medium. Total chlorophyll was determined as described [18].

Treatment of chloroplasts with phenylglyoxal was carried out at 25°C in a reaction medium (1 ml) containing 50 mM borate buffer (pH 7.8), appropiate concentration of phenylglyoxal and chloroplasts (100–250 μg chlorophyll). Aliquots were removed

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at adequate times and transferred to the reaction medium for photophosphorylation, Mg–ATPase or P_i–ATP exchange activities. Solutions of phenylglyoxal in 50 mM borate buffer (pH 7.8) were freshly prepared for each experiment.

Cyclic photophosphorylation was determined as described [17]. The reaction medium (1 ml) was the same used for suspending the chloroplasts with addition of 2 mM ADP, 3 mM sodium phosphate containing 1×10^6 cpm ^{32}P, 33 μM phenazine methosulfate and chloroplasts (10 μg chlorophyll).

The light- and dithioerythritol-triggered, Mg-dependent ATPase and ATP–P_i exchange reactions of chloroplasts were determined as described [19,20].

Spinach coupling factor 1 was prepared and purified according to [21]. Aliquots of enzyme, stored at 4°C in 2 M $(NH_4)_2$ SO_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–ATPase of CF_1 was performed as described [21] except that the reaction medium was 50 mM borate buffer (pH 7.8).

Protein was determined according to [22], with bovine serum albumin as the standard.

Modification of ATPase activity of CF_1 with phenylglyoxal or 2,3-butanedione was carried out at 25°C in a reaction medium (1 ml) containing 50 mM borate buffer (pH 7.8), appropiate concentration of the reagents and 100 μg of trypsin-activated ATPase. At adequate times aliquots of 3 μg enzyme were removed and transferred to the reaction medium for Ca–ATPase activity. The assay of ATPase was performed essentially as described [8].

Dithioerythritol, phenylglyoxal, trypsin, trypsin inhibitor and nucleotides were obtained from Sigma (USA). 2,3-Butanedione was obtained from BDH Chemicals (England).

All other chemicals were of analytical grade.

3. Results and discussion

Chemical modification of spinach chloroplasts by incubation with several phenylglyoxal concentrations resulted in inactivation of its cyclic photophosphorylation and the light and DTE triggered ATP–P_i exchange, and Mg–ATPase activities. Figure 1 shows that inactivation of the three reactions followed

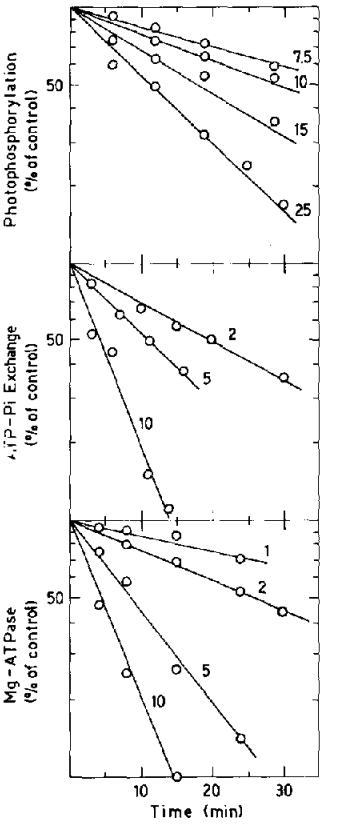

Fig.1. Inactivation of cyclic photophosphorylation. ATP–P_i exchange and Mg–ATPase activities of spinach chloroplasts by phenylglyoxal. Semilogarithmic plots of the remaining activities after treatment with several phenylglyoxal concentrations (numerals on the slopes, mM) for the times stated. Experimental conditions were as described in the text.

apparent first order kinetics until, with the higher concentrations of inhibitor, less than 10% of the initial activities remained. Complete inhibition was achieved with longer incubation times.

Treatment of chloroplasts with the arginine reagent 2,3-butanedione resulted also in a similar inactivation of photophosphorylation except that inactivation was preceded by a short stimulation and required longer incubation times (not shown).

The reaction orders with respect to phenylglyoxal for the the inactivation of photophosphorylation, ATP–P_i exchange and Mg–ATPase activities were determined from a plot of the log of the pseudo-first order reaction constants (k') for inhibition versus the

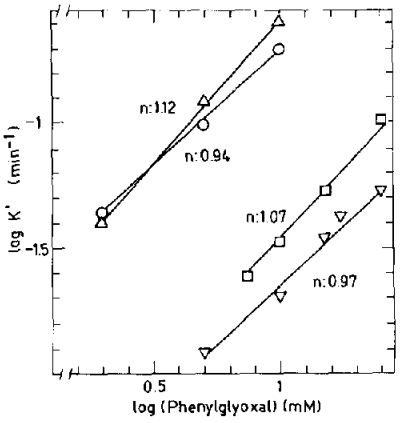

Fig.2. Plot of the log of pseudo-first order rate constants (k') of inactivation of photophosphorylation (□ – □), chloroplast Mg–ATPase (○ – ○), ATP–P_i exchange reaction (△ – △) and CF_1 (Ca–ATPase (▽–▽) versus the log of phenylglyoxal concentrations. n: slopes of lines indicating reaction orders with respect to inhibitor concentration.

log of phenylglyoxal concentrations (fig.2) as used [8,15,16,23–26]. The reaction orders obtained ranged from 0.94 to 1.12, suggesting that modification by phenylglyoxal of only one arginine residue per active site produced the observed inhibition of photophosphorylation, ATP–P_i exchange and Mg–ATPase reactions in chloroplasts.

The Ca–ATPase activity of soluble spinach CF_1 was also inactivated by phenylgyoxal and the reaction order obtained was 0.97 (fig.2). This result was in agreement with a similar one achieved with CF_1 and 2,3-butanedione [8].

The second order rate constants of the inhibition of photophosphorylation, ATP–P_i exchange and Mg–ATPase reactions by incubation of chloroplasts with phenylglyoxal were calculated from a plot of k' versus phenylglyoxal concentrations and the k values obtained were 3.6, 21 and 24 M^{-1} min^{-1}, respectively. It can be seen that the ATP–P_i exchange and Mg–ATPase reactions were more sensitive to the reagent than photophosphorylation. This fact, together with the observation that chemical modification of a single arginine per active site in spinach chloroplasts leads to inhibition of the three reactions suggests that the arginine involved in the ATP–P_i exchange and Mg–ATPase reactions is different from the one involved in photophosphorylation.

ATP–P_i exchange activity of an ATP synthetase preparation from beef heart mitochondria is more susceptible than the ATPase activity of the same preparation to the arginine-binding reagents butanedione and phenylglyoxal [27]. This result agrees with a similar observation in beef heart submitochondrial particles [15]. Both research groups suggested that the mitochondrial ATP–P_i exchange activity involves a second, more susceptible, arginine residue. However, no determination of the effects of the arginine reagents on oxidative phosphorylation was reported.

The location in chloroplasts of the arginyl residue(s) essential for ATP synthesis and hydrolysis should be in the bound CF_1 since:

(i) Modification of only one arginine per active site in chloroplasts or in soluble CF_1 leads to complete inhibition of ATP synthesis and hydrolysis;

(ii) As shown in table 1, modification of soluble native CF_1 by either phenylglyoxal or 2-3 butanedione resulted in a loss of its coupling activity similar to the loss in its ATPase activity.

(iii) Adenine nucleotides protected both processes as described below.

Jagendorf et al. have recently found (Biochim. Biophys. Acta, in press) that photophosphorylation and the ATPase of chloroplasts were inhibited by butanedione and phenylglyoxal behaving as specific energy transfer inhibitors. They suggested that the reagents attack arginine residues of the coupling factor.

Table 2 shows the results of a study of the protection afforded by several nucleotides against inactivation by phenylglyoxal of photophosphorylation in chloroplasts or Ca–ATPase activity of soluble CF_1. Adenine and guanine nucleotides protected both reactions, while CTP, CDP, UDP, adenosine, P_i and PP_i, at a concentration of 10 mM, did not. ATP and GTP afforded a higher protection of the ATPase activity of CF_1 than of chloroplast photophosphorylation. The protection by nucleotides suggests that the essential arginine residue participates in the binding of the nucleotides to the catalytic site(s) of CF_1 in agreement with a similar role suggested in other enzymes [11–16].

In other experiment, chloroplasts were incubated with 10 mM phenylglyoxal in the presence of 25 mM ATP and photophosphorylation and the light- and DTE-triggered ATPase activities were determined at

Table 1
Effect of butanedione or phenylglyoxal treatment of CF_1 on its coupling and ATPase activities

Additions to EDTA–chloroplasts	Photophosphorylation (μmol.h^{-1}mg^{-1}chl.)	Ca–ATPase activity of CF_1 added (μmol.min^{-1}mg^{-1})
None	11.3	–
CF_1	57.2	15.9
CF_1–Phenylglyoxal	33.1	7.8
CF_1–Butanedione	17.5	6.3

EDTA-particles from spinach chloroplasts were obtained essentially as described [28]. The reconstitution of EDTA-particles was performed at 0°C for 20 min in a reaction mixture (0.9 ml) containing 10 μmol NaCl, 10 μmol $MgCl_2$, 20 μmol tricine (pH 8), 1 mg/ml bovine albumin, EDTA-particles (50 μg chlorophyll) and 100 μg CF_1. After the incubation, 0.1 ml reaction mixture containing 10 μmol NaCl, 10 μmol $MgCl_2$, 3 μmol P_i (containing 2×10^6 cpm ^{32}P), 2 μmol ADP, 50 μmol phenazine methosulphate and 20 μmol tricine were added and photophosphorylation was determined as described [17]

Aliquots of CF_1 (1 mg) were modified by 25 mM phenylglyoxal or 2,3-butanedione as described in the text during 30 min and 60 min, respectively. After the incubation the excess of reagent was eliminated by passing through a column of Sephadex G-50 (0.8×15 cm) equilibrated with 50 mM borate buffer (pH 7.8)

Table 2
Protection by nucleotides of ATP synthesis and hydrolysis against inactivation by phenylglyoxal

Additions during preincubation with phenylglyoxal	k' (min^{-1})	
	ATP hydrolysis (CF_1)	ATP synthesis
None	0.051	0.095
ATP 5 mM	0.036	0.080
ATP 10 mM	0.026	0.064
ATP 25 mM	0.026	0.062
ADP 5 mM	0.038	0.074
ADP 10 mM	–	0.064
ADP 25 mM	–	0.051
AMP 10 mM	0.040	0.071
GTP 25 mM	0.028	0.069
GDP 10 mM	0.034	0.071

The pseudo-first order constants of inactivation by 25 mM phenylglyoxal, with the additions stated, of the Ca–ATPase activity of soluble CF_1 and cyclic photophosphorylation in spinach chloroplasts were determined as described in the text

different times in the same aliquots of chloroplast suspensions. The protection afforded by ATP against phenylglyoxal inactivation of the ATPase activity of chloroplasts was the same as reported in table 2 for the ATPase of soluble CF_1 (k' was diminished by 50% from 0.167–0.083 by ATP) and twice as high as the protection of photophosphorylation (k' was diminished by 25% from 0.036–0.027 by ATP) when measured simultaneously in the same chloroplast preparation.

Sites for ATP synthesis and hydrolysis in chloroplasts may be different based on the use of 1,N^6-ethenoa-denosine di- and triphosphate as substrates [29]. Mitochondrial oxidative phosphorylation and ATPases has been suggested [30,31].

The differences in susceptibility to phenylglyoxal of photophosphorylation and ATPase and the different degree of protection afforded by adenine nucleotides might be related to the postulated different catalytic sites for these reactions in chloroplasts. We have recently found evidence favorable to the existence of different catalytic sites of ATP synthesis and hydrolysis in *Rhodospirillum rubrum* chromatophores (Vallejos, Lescano and Lucero, in preparation).

CF_1 has multiple binding sites for adenine nucleotides [1] and it is possible that each one has an arginine residue.

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