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SULPHYDRYL GROUPS IN PHOTOSYNTHETIC ENERGY CONSERVATION

IV. INHIBITION OF THE ATPase OF CHLOROPLAST COUPLING FACTOR 1 BY SULPHYDRYL REAGENTS

RUBÉN H. VALLEJOS, RICARDO A. RAVIZZINI and CARLOS S. ANDREO

Departamento de Bioquímica, Facultad de Ciencias Bioquímicas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario (Argentina)

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SUMMARY

1. *o*-Iodosobenzoate and 2,2′-dithio bis-(5-nitropyridine) inhibited by about fifty per cent the ATPase activity of heat-activated chloroplast coupling factor 1 only when present during the heating but were without effect when added before or after the activation. Reversion of this inhibition was only obtained by a second heat treatment with 10 mM dithioerythritol.

2. The inhibition of the Ca^{2+}-ATPase of coupling factor 1 by *o*-iodosobenzoate or 2,2′-dithio bis-(5-nitropyridine) was not additive with similar inhibitions obtained with the alkylating reagents iodoacetamide and *N*-ethylmaleimide.

3. The heat-activated ATPase of *o*-iodosobenzoate-treated coupling factor 1 had a higher K_m for ATP, without modification of V. The modified enzyme was desensitized against the allosteric inhibitor ADP.

INTRODUCTION

The soluble latent ATPase of chloroplasts or coupling factor 1 plays a central role in the photosynthetic formation of ATP. It has a molecular weight of 325 000 [1] and is made up of five different subunits with the following molecular weights: α, 59 000; β, 56 000; γ, 37 000; δ, 17 500 and ε, 13 000 (2). Coupling factor 1 contains 12 half-cystines per mole, 8 of which are free thiol groups and 4 are involved in 2 disulphide bridges [3].

The purified coupling factor 1 of spinach chloroplast has a latent ATPase which may be activated by trypsin [4], heating [3, 4] or thiol compounds [3, 5].

Only two sulphydryl groups are available to sulphydryl reagents in coupling factor 1 [3] but after heat activation of the ATPase two more thiol groups of the γ subunit become exposed [3, 6]. Blocking of these thiol groups by iodoacetamide or

Abbreviation: DTNP, 2,2′-dithio bis-(5-nitropyridine).

N-ethylmaleimide did not affect the emerging ATPase activity ([6, 7] but see also ref. 3).

We have recently shown [8, 9] a light-dependent inhibition of photophosphorylation, coupled electron transport and ATPase activity of spinach chloroplasts by the sulphydryl reagents 2,2'-dithio bis-(5-nitropyridine) (DTNP) and *o*-iodosobenzoate. The inhibition was attributed to the oxidation of vicinal dithiols exposed by a conformational change of coupling factor 1 induced by light. These results were in agreement with previous reports by McCarty et al. [10] of similar light-dependent effects obtained with the alkylating agent *N*-ethylmaleimide.

In this paper we show that *o*-iodosobenzoate, and DTNP, as well as other sulphydryl reagents, are able to inhibit the heat-activated ATPase of coupling factor 1 only when present during the process.

RESULTS

Inhibition of the Ca^{2+}-ATPase activity of chloroplast coupling factor 1 by sulphydryl reagents

Table I, Expts. 1 and 2, shows that the ATPase activity of heat-activated coupling factor 1 was not affected by 5 mM *o*-iodosobenzoate or 300 μM DTNP added after the activation or by incubation of the reagents with the enzyme for 1 h

TABLE I

INHIBITION OF THE Ca^{2+}-ATPase ACTIVITY OF CHLOROPLAST COUPLING FACTOR 1 BY *o*-IODOSOBENZOATE AND DTNP

In Expt. 1, aliquots of latent ATPase (1 mg/ml) were incubated in 40 mM Tris · HCl, 2 mM EDTA (pH 8) for 1 h at 25 °C with the additions stated. Then the protein was passed through a 0.8 × 15 cm Sephadex G-50 column, and the Ca^{2+}-ATPase of coupling factor 1 was activated and measured as described in the text. In Expt. 2 the incubation described for Expt. 1, 1 h at 25 °C, was performed after the heat activation in the presence of 40 mM ATP. In Expts. 3 and 4 the additions stated were present during the activation. When added, *o*-iodosobenzoate was 5 mM; DTNP, 300 μM; and dithioerythritol, 10 mM.

Expt.	Additions	Ca^{2+}-ATPase (μmol $P_i \cdot min^{-1} \cdot mg^{-1}$)
1	None	20.0
	o-Iodosobenzoate before heating	19.5
	DTNP before heating	19.5
2	None	18.1
	o-Iodosobenzoate after heating	19.0
	DTNP after heating	16.0
3	None	20.0
	o-Iodosobenzoate during heating	11.6
	DTNP during heating	9.4
4	Dithioerythritol	36.0
	Dithioerythritol and *o*-iodosobenzoate during heating	35.6
	Dithioerythritol and DTNP during heating	34.6

TABLE II

REVERSAL OF ATPase INHIBITION BY *o*-IODOSOBENZOATE OR DTNP

Activation of Ca^{2+}-ATPase of chloroplast coupling factor 1 by heating in the absence or in the presence of *o*-iodosobenzoate or DTNP was carried out as described in the text. Then, a second treatment of coupling factor 1 was performed as indicated followed by determination of the ATPase activity as described in the text. Numbers in parenthesis indicate inhibition per cent.

Second treatment of coupling factor 1	Ca^{2+}-ATPase (μmol $P_i \cdot min^{-1} \cdot mg^{-1}$)		
	Controls	*o*-Iodosobenzoate-treated	DTNP-treated
None	18.9	11.9 (37)	9.4 (50)
60 min at 25 °C in the presence of 10 mM dithioerythritol	26.1	16.2 (38)	13.0 (50)
4 min at 63 °C in the presence of 10 mM dithioerythritol	32.8	33.0	32.5
4 min at 63 °C after removal of excess of reagent of the first treatment	16.1	9.8 (39)	7.9 (51)

followed by passing the mixture through a Sephadex G-50 column before activation. These sulphydryl reagents did not affect the ATPase activated by trypsin treatment added either before or after activation (not shown). However, when *o*-iodosobenzoate or DTNP were present during heat-activation of the enzyme they diminished the resulting Ca^{2+}-ATPase activity by about 40–50 % (Table I, Expt. 3). When the heating was carried out in the presence of 10 mM dithioerythritol the ATPase activity was higher [3] and dithioerythritol prevented inhibition by DTNP and *o*-iodosobenzoate (Table I, Expt. 4).

Incubation of chloroplast coupling factor 1 with 10 mM dithioerythritol for 1 h after heat-activation of the former in the presence of *o*-iodosobenzoate or DTNP did not result in reversion of the resulting inhibition (Table II, line 2) although it stimulated the control and the treated ATPases. Complete reversion of the inhibition of the ATPase by the sulphydryl reagents was achieved when coupling factor 1 was

TABLE III

EFFECTS OF SULPHYDRYL REAGENTS OF THE ATPase ACTIVITY OF COUPLING FACTOR 1

The first treatment of coupling factor 1 with 5 mM *o*-iodosobenzoate was performed as described in the text, then the protein was passed through a 0.8 × 15 cm Sephadex G-50 column and a second treatment at 63 °C for 4 min in the presence of 40 mM ATP and with the additions stated was performed.

Additions during second heating	Ca^{2+}-ATPase (μmol $P_i \cdot min^{-1} \cdot mg^{-1}$)	
	Control	*o*-Iodosobenzoate-treated
None	18.3	11.2
Iodoacetamide, 10 mM	13.7	10.8
N-ethylmaleimide, 10 mM	5.5	10.2

heated a second time at 63 °C for 4 min but in the presence of 10 mM dithioerythritol (Table II, line 3). A second heat treatment in the absence of dithioerythritol did not reverse the inhibition (cf. lines 4 and 1, Table II).

The ATPase activity of coupling factor 1 was not affected by sulphydryl alkylating agents, even though they blocked the two accessible thiol groups of coupling factor 1 or the two additional groups that become accessible after heat activation [6, 7]. However, iodoacetamide and N-ethylmaleimide were able to inhibit the ATPase of coupling factor 1 if present during heating (Table III, line 2 and 3). *N*-Ethylmaleimide consistently inhibited more than iodoacetamide (50 % inhibition was obtained with 2.5 mM *N*-ethylmaleimide). The partial inhibitory effects of the alkylating and oxidating sulphydryl reagents were not additive as shown in Table III, second column, for *o*-iodosobenzoate. Similar results were obtained with DTNP or with the order of treatment inverted. These results suggest that the same sulphydryl groups are affected by all these reagents in spite of different reaction mechanisms. For instance, pretreatment with *o*-iodosobenzoate prevented the inhibition by *N*-ethylmaleimide probably because the sulphydryl groups affected by the latter were already oxidized to disulfides by the former inhibitor (Table III, line 3).

o-Iodosobenzoate oxidizes vicinal dithiols to disulfide [11] and DTNP may also oxidize them [8]. As discussed for the light-dependent effects of these reagents in chloroplasts [8, 9] the resulting disulfide bridge(s) may be intra or interpeptidic considering the oligomeric nature of coupling factor 1. The subunit mobility of coupling factor 1 in sodium dodecyl sulfate polyacrylamide electrophoresis carried out without β-mercaptoethanol did not change with *o*-iodosobenzoate or DTNP treatment (experiments not shown) suggesting that the disulfide bridge is intrapeptidic.

Kinetic properties of o-iodosobenzoate-inhibited ATPase

Fig. 1 shows the kinetics of the ATPase activity of chloroplast coupling factor 1 with or without treatment with 5 mM *o*-iodosobenzoate. A Lineweaver-Burk plot of

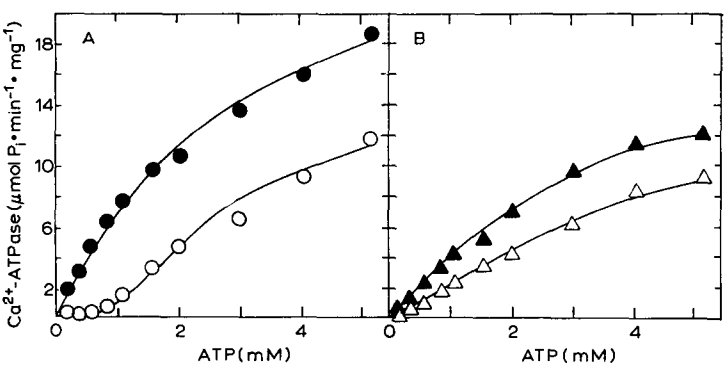

Fig. 1. Effect of *o*-iodosobenzoate treatment of coupling factor 1 on the allosteric inhibition of Ca^{2+}-ATPase activity by ADP. The heat activation and *o*-iodosobenzoate treatment of coupling factor 1 in the absence (●, ○) or in the presence (▲, △) of 5 mM *o*-iodosobenzoate was performed as described in the text. The Ca^{2+}-ATPase assay was as described in the text. A, untreated coupling factor 1. B: *o*-iodosobenzoate-treated coupling factor 1. Closed symbols, controls; open symbols, 1 mM ADP present.

the data showed that the sulphydryl reagent decreased the affinity of the enzyme for ATP (the K_m increased from 1.85 to 6.5 mM), without modifying the V.

Nelson et al. [12] have shown that ADP is an allosteric inhibitor of the ATPase of coupling factor 1. This is clearly shown in Fig. 1A where it can be observed that the hyperbolic curve for ATP was changed into a sigmoidal one when 1 mM ADP was present. Treatment of coupling factor 1 with *o*-iodosobenzoate in the way described changed this kinetic behavior as shown in Fig. 1B, since in the presence of ADP, the ATP saturation curve was considerably less sigmoidal and the inhibition by ADP was diminished.

Hill plots of these results show that, as previously described [12], the apparent n increased from 0.99 to 1.75 in the presence of 1 mM ADP. On the other hand, with the *o*-iodosobenzoate-treated enzyme the apparent n values were nearly the same.

In an experiment where the ADP concentrations were varied from 0.35 to 3 mM it was found that the concentration of ADP that gave 50 % inhibition of the ATPase in the presence of 5 mM ATP was raised from 0.85 mM in the untreated control to 1.90 mM in *o*-iodosobenzoate-treated coupling factor 1.

DISCUSSION

The sulphydryl groups of coupling factor 1 have been related to the activation of the ATPase since sulphydryl groups of the γ subunit become exposed after heat activation and the ATPase activity is greater in the presence of thiol compounds [3]. Activation of the ATPase in chloroplasts required light and thiol compounds [13].

The results presented in Tables I and III showing that sulphydryl reagents only inhibited the heat-activated ATPase of coupling factor 1 when present during the activation, explain the previously reported lack of effect [6, 7] and suggest that the thiol groups involved are inside the coupling factor molecule. The requirement of a second heat treatment in the presence of dithioerythritol for reversion of *o*-iodosobenzoate inhibition reinforces this conclusion (Table II).

It has been shown that light induces profound conformational changes in chloroplast-bound coupling factor 1 which may result in the incorporation of tritium [14, 15], the oxidation of vicinal dithiols [8, 9], the alkylation of sulphydryl groups [10, 16] or changes in the fluorescence emission of fluorescamine-labelled ATPase [17]. The conformational changes are of such nature as to expose groups deeply buried in the molecule as suggested by the observations that coupling factor 1 may be solubilized from the membrane without losing the incorporated tritium [15] and that reversion of the inhibition associated with oxidation of dithiols required a second illumination of the chloroplasts to make the disulfide bridges accessible to dithioerythritol [8, 9].

The thiol group(s) exposed by a light-induced conformational change of membrane-bound coupling factor 1 may be the same that reacted with *o*-iodosobenzoate, DTNP or *N*-ethylmaleimide during heat activation of soluble coupling factor 1 as suggested by the partial inhibitions obtained in both systems and the lack of additivity of the inhibitions (ref. 9 and Table III). The subunit localization of the involved thiols is not yet clear, although the γ subunit is pointed out by the light-dependent incorporation of labelled *N*-ethylmaleimide [16]. On the other hand Deters et al. [7] have suggested that the least accessible thiol groups of coupling factor 1 are in the β subunit.

The kinetic properties of *o*-iodosobenzoate-treated chloroplast coupling factor 1 are quite similar to those found by Datta et al. [18] with the ATPase of permanganate-treated chloroplasts and of chloroplasts preincubated in light with *o*-iodosobenzoate (unpublished observations). The ATPase of purified coupling factor 1 was desensitized by *o*-iodosobenzoate treatment for the allosteric inhibition by ADP. This suggests that the allosteric binding sites are modified by the reagents. The localization of these sites has been proposed to be on the α subunit of coupling factor 1 [19].

EXPERIMENTAL

Purified coupling factor 1 of spinach chloroplasts was prepared according to Lien and Racker [20]. The step of sucrose density gradient centrifugation was omitted. Preparations with a specific Ca^{2+}-ATPase activity after heat activation of about 20 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ 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 $10000 \times g$ and dissolved with 0.5 ml of 40 mM Tris · HCl (pH 8), and 2 mM EDTA. The enzyme (0.5 to 1 mg per ml) was desalted on a Sephadex G-50 column (0.8 cm × 15 cm) equilibrated with the same solution. Heat activation was carried out at 63 °C for 4 min in a medium containing 40 mM Tris · HCl (pH 8), 2 mM EDTA and 40 mM ATP, followed by cooling with running tap water.

ATPase activity was measured at 37 °C in a final volume of 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 μg of heat activated coupling factor 1. After 10 min, the reaction was stopped by addition of 0.1 ml of trichloroacetic acid 50 % (w/v). In the supernatants the P_i liberated was determined colorimetrically according to Sumner [21].

Protein was determined according to Lowry et al. [22].

Sodium dodecyl sulfate gel electrophoresis was performed as described by Weber and Osborn [23].

DTNP, *o*-iodosobenzoate, iodoacetamide, *N*-ethylmaleimide, dithioerythritol and nucleotides were obtained from Sigma Chemical Co (U.S.A.). All other chemicals were of analytical grade.

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REFERENCES

1 Farron, F. (1970) Biochemistry 9, 3823–3828
2 Lien, S., Berzborn, R. J. and Racker, E. (1972) J. Biol. Chem. 247, 3520–3524
3 Farron, F. and Racker, E. (1970) Biochemistry 9, 3829–3836
4 Vambutas, V. K. and Racker, E. (1965) J. Biol. Chem. 240, 2660–2667
5 McCarty, R. E. and Racker, E. (1968) J. Biol. Chem. 243, 129–137
6 Cantley, L. C. and Hammes, G. G. (1976) Biochemistry 15, 9–14

7 Deters, D. W., Racker, E., Nelson, N. and Nelson, H. (1975) J. Biol. Chem. 250, 1041–1047
8 Andreo, C. S. and Vallejos, R. H. (1976) Biochim. Biophys. Acta 423, 590–601
9 Vallejos, R. H. and Andreo, C. S. (1976) FEBS Lett. 61, 95–99
10 McCarty, R. E., Pittman, P. R. and Tsuchiya, Y. (1972) J. Biol. Chem. 247, 3048–3051
11 Webb, L. (1966) Metabolic Inhibitors, Vol. II, pp. 701–728, Academic Press
12 Nelson, N., Nelson, H. and Racker, E. (1972) J. Biol. Chem. 247, 6506–6510
13 Petrack, B. and Lipmann, F. P. (1961) in Light and Life (McElroy, W. D. and Glass, H. B. eds.), pp. 621–630. The John Hopkins Press, Baltimore
14 Ryrie, I. J. and Jagendorf, A. T. (1971) J. Biol. Chem. 246, 3771–3774
15 Ryrie, I. J. and Jagendorf, A. T. (1972) J. Biol. Chem. 247, 4453–4459
16 McCarty, R. E. and Fagan, J. (1973) Biochemistry 12, 1503–1507
17 Kraayenhof, R. and Slater, E. C. (1974) Proc. Third Intern. Congr. Photosynthesis (Avron, M., ed.), Vol. II, pp. 985–996, Elsevier, Amsterdam
18 Datta, D. B., Ryrie, I. J. and Jagendorf, A. T. (1974) J. Biol. Chem. 249, 4404–4411
19 Cantley, L. C. and Hammes, G. G. (1975) Biochemistry 14, 2976–2981
20 Lien, S. and Racker, E. (1974) Methods in Enzymology (San Pietro, A., ed.), Vol. XXIII A, pp. 547–555, Academic Press, New York
21 Sumner, J. B. (1944) Science 100, 413–414
22 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
23 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412