MODIFICATION OF ESSENTIAL CARBOXYL RESIDUES IN CHLOROPLAST COUPLING FACTOR 1

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1. Introduction

Chemical modification of enzymes has increasingly been used to identify amino acid residues essential for catalytic activities.

Woodward's reagent K was used to modify carboxyl groups of carboxypeptidase A [1]. Since the structure of this reagent includes an aromatic ring and negative charge that might resemble ATP it was used successfully to show the presence of an essential carboxyl group in yeast phosphoglycerate kinase [2]. It was reported that the carboxyl group of an aspartic residue may be in the ATP binding site of adenylate kinase [3]. Several arginyl residues were also shown around this site.

The H^+-ATPases of bacteria, chloroplasts and mitochondria have essential arginyl residues in their nucleotide binding sites [4–6]. The soluble mitochondrial and *Escherichia coli* ATPases [7–12] are inactivated by carboxyl reagents such as *N*-ethoxy carbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) and dicyclohexylcarbodiimide (DCCD). The latter reagent binds to the β subunit of both enzymes.

The present paper reports on the probing of the carboxyl residues of spinach chloroplasts coupling factor 1 (CF_1) by Woodward's reagent K. It is shown that photophosphorylation in chloroplasts and the ATPase activities of both chloroplasts and soluble CF_1 were inactivated when one carboxyl residue per active site was modified. ADP and ATP protected against modification.

2. Experimental

Chloroplasts were isolated as previously described [13] from spinach leaves (*Spinacea oleracea* L.) obtained from the market and finally resuspended in 40 mM Tricine–NaOH (pH 7.8), 175 mM NaCl and 5 mM $MgCl_2$.

CF_1 was purified from spinach chloroplasts and its latent ATPase was activated by heat and assayed as described [14]. Photophosphorylation [13], electron transport [15], light- and DTE-triggered ATPase [16], protein [17], and chlorophyll concentration [18] were assayed by published methods.

Woodward's reagent K, ADP, ATP, methylviologen and dithioerythritol were obtained from Sigma. All other chemicals used were of analytical grade.

3. Results and discussion

Treatment of spinach chloroplasts with Woodward's reagent K resulted in a rapid inactivation of noncyclic photophosphorylation (fig.1). Initially the kinetics of inactivation were of apparent first order but later the reagent concentrations were presumably reduced by hydrolysis [1]. With higher concentrations of the reagent the inactivation was complete. Cyclic photophosphorylation catalyzed by phenazine methosulfate was also inhibited (not shown).

Modification of chloroplasts with Woodward's reagent K affected neither the basal nor the uncoupled electron transport from water to methylviologen (table 1) but the coupled electron transport was inhibited to the basal level. Thus, inactivation by this reagent is similar to the effect of energy transfer inhibitors. The site of modification is not in the electron transport components and may be on the H^+-ATPase.

Energy transfer inhibitors may inhibit the ATPase complex by acting on either the membrane compo-

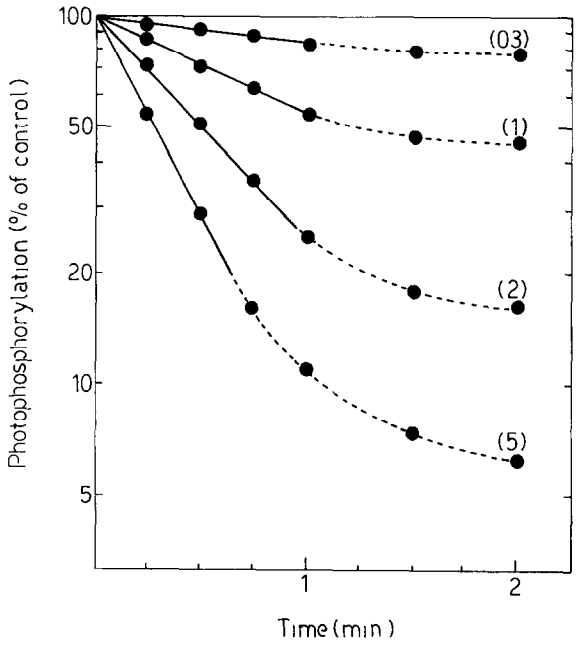

Fig.1. Effect of Woodward's reagent K on spinach chloroplast non-cyclic photophosphorylation. Chemical modification of chloroplasts (0.5 mg chl/ml) was carried out in 40 mM Tricine-NaOH (pH 7.8), 175 mM NaCl and 5 mM $MgCl_2$ at 25°C with the concentrations of Woodward's reagent K stated (mM). At the times stated aliquots of 20 μl were diluted in the reaction medium and photophosphorylation associated with water-methylviologen electron transport was measured as described in the text.

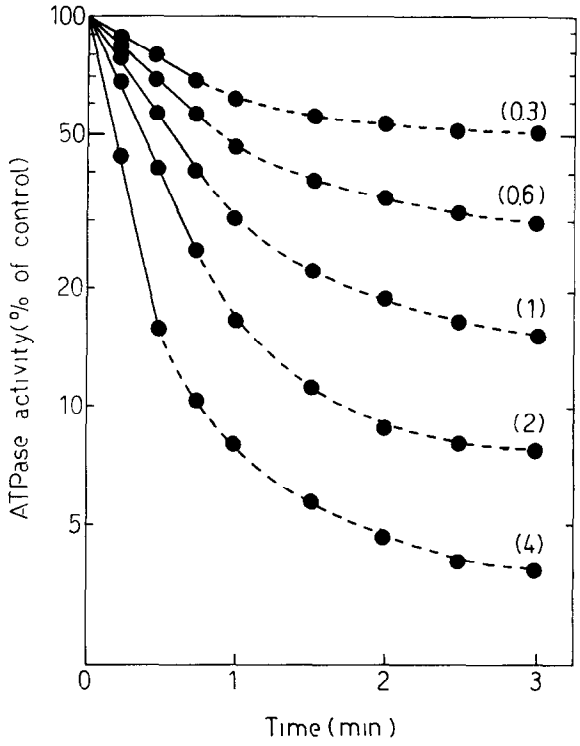

Fig.2. Effect of Woodward's reagent K on the ATPase activity of CF_1. Modification of heat-activated CF_1 (0.5 mg/ml) with the stated concentration (mM) of Woodward's reagent K was carried out in 40 mM Tricine-NaOH (pH 8). At the times stated aliquots of CF_1 were diluted 100 times in the reaction mixture for ATPase activity.

nent (CF_o) like DCCD or Discarine B, or on the soluble component (CF_1) like phloridzin, Dio-9 or spegazinine [19–21]. The second type of inhibitor affected both the membrane bound and the soluble ATPase activities. That is also the case for Woodward's reagent K (fig.2 and table 2).

Fig.2 shows that the ATPase activity of soluble CF_1 was also inhibited by Woodward's reagent K. Similar

Table 1
Effect of Woodward's reagent K on the photosynthetic electron transport

Chloroplast treatment	Electron transport (μmol O_2/h/mg chl)		
	Basal	Coupled	Uncoupled
None	41	80	180
Woodward's reagent K, 0.3 mM	41	69	178
Woodward's reagent K, 1 mM	40	53	178
Woodward's reagent K, 5 mM	40	40	165

Spinach chloroplasts were modified for 20 min with the stated concentrations of Woodward's reagent K as described in the legend to fig.1. Then electron transport from water to methylviologen was measured in basal, coupled (2 mM ADP and 2 mM P_i present), and uncoupled (10 mM methylamine present) conditions

Table 2
Protection by adenine nucleotides against inactivation of photophosphorylation and ATPase by Woodward's reagent K

Additions during modification	ATP synthesis (Half times of inactivation)	Mg-ATPase	Ca-ATPase
None	0.8	0.5	0.5
ADP	1.5	0.8	1.5
ATP	1.3	1.0	1.3
$MgCl_2$	0.7	0.5	0.5

Chloroplasts or CF_1 were modified with 1 mM Woodward's reagent K with the additions stated. Chloroplast modification was stopped at different times by adding to aliquots 0.1 vol. of 1 M sodium acetate. Then photophosphorylation and light- and DTE-triggered Mg-ATPase activities were measured. Heat-activated CF_1 was modified as described in the legend to fig.2 and its Ca-ATPase activity determined. ADP, ATP and $MgCl_2$, when present during modification, were 20 mM

results were obtained when modification was carried out at pH 6, instead of 8, where both inactivation and hydrolysis were slower.

The order of reaction with respect to the inhibitor concentration was 0.85, 0.88 and 0.96 for the ATPase inactivated at pH 8, the ATPase inactivated at pH 6 and photophosphorylation, respectively (fig.3). This

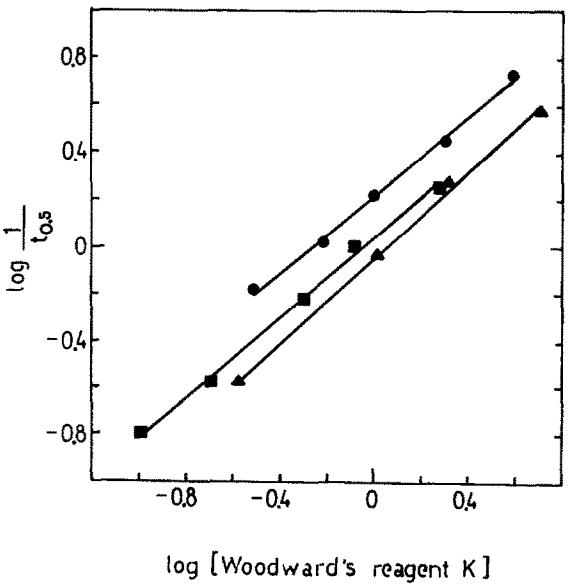

Fig.3. Apparent order of reaction (n) with respect to Woodward's reagent K concentration. Half times of inactivation ($t_{0.5}$) were calculated from plots like figs.1 and 2 for photophosphorylation (▲–▲) and ATPase activity of CF_1 measured at pH 8 (●–●) or at pH 6 (■–■).

suggests the involvement of one carboxyl residue per active site of CF_1 [22].

The light- and DTE-triggered ATPase of chloroplasts was also inactivated by Woodward's reagent K (table 2). Adenine nucleotides protected photophosphorylation and ATPases from both chloroplasts and soluble CF_1 (table 2). ADP or ATP (20 mM) afforded similar protection while Ca^{2+} or Mg^{2+} neither protected nor affected protection by nucleotides. These results suggest that the essential carboxyl residue may be in the nucleotide-binding site of CF_1.

The purified ATPase from the thermophilic bacterium PS-3 (TF_1) was also inactivated by Woodward's reagent K [23]. The essential carboxyl residue of TF_1 was protected by adenine nucleotides and may be located in the β subunit.

Mitochondrial F_1 was inhibited by different carboxyl reagents [9,10]. At variance with the results described in this paper for CF_1, F_1 was protected by Mg^{2+} but little or none by nucleotides.

DCCD is known [19] to inhibit photophosphorylation and to restore proton uptake in CF_1-depleted membranes by acting on CF_0. ADP and P_i did not prevent its effects. It has recently been reported that the ATPase activity of soluble CF_1 was also inhibited by *N*-cyclohexyl-*N'*-*p*(4-methyl morpholyl-ethyl carbodiimide [24] and by DCCD [25].

Woodward's reagent K does not seem to act on CF_0 like DCCD since it was unable to stimulate proton uptake in EDTA particles (data not shown), the inhibition of photophosphorylation was prevented by adenine nucleotides and was similar to the inactivation of soluble CF_1. Moreover we found that inactivation

of CF_1 by 250 μM DCCD was protected by 20 mM $CaCl_2$ (half time of inactivation was increased from 20 to 70 min by 20 mM $CaCl_2$) but not by 20 mM ATP or ADP, suggesting that the carboxyl residue modified by Woodward's reagents K is different from the residue modified by DCCD.

In conclusion modification of chloroplasts and CF_1 by Woodward's reagent K suggests that CF_1 contains an essential carboxyl residue which may be located near the nucleotide binding site.

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References

[1] Pétra, P. H. (1971) Biochemistry 10, 3163–3177.
[2] Brake, A. J. and Weber, B. H. (1974) J. Biol. Chem. 249, 5452–5457.
[3] Pai, E. F., Sachsenheimer, W., Schirmer, R. H. and Schulz, G. E. (1977) J. Mol. Biol. 114, 37–45.
[4] Vallejos, R. H., Lescano, W. I. M. and Lucero, H. A. (1978) Arch. Biochem. Biophys. 190, 578–584.
[5] Andreo, C. S. and Vallejos, R. H. (1977) FEBS Lett. 78, 207–210.
[6] Marcus, F., Schuster, S. M. and Lardy, H. A. (1976) J. Biol. Chem. 251, 1775–1780.
[7] Penefsky, H. S. (1977) J. Biol. Chem. 242, 5789–5795.
[8] Beechey, R. B., Hubbard, S. A., Linnet, P. E., Mitchell, A. D. and Munn, E. A. (1975) Biochem. J. 148, 533–537.
[9] Pougeois, R., Satre, M. and Vignais, P. V. (1978) Biochemistry 17, 3018–3023.
[10] Pougeois, R., Satre, M. and Vignais, P. V. (1979) Biochemistry 8, 1408–1412.
[11] Kozlov, I. A. and Skulachev, V. P. (1977) Biochim. Biophys. Acta 463, 29–89.
[12] Satre, M., Lunardi, J., Pougeois, R. and Vignais, P. V. (1979) Biochemistry 18, 3134–3140.
[13] Vallejos, R. H. (1973) Biochim. Biophys. Acta 292, 193–196.
[14] Andreo, C. S., Ravizzini, R. A. and Vallejos, R. H. (1979) Biochim. Biophys. Acta 547, 370–379.
[15] Andreo, C. S. and Vallejos, R. H. (1976) Biochim. Biophys. Acta 423, 590–601.
[16] Andreo, C. S. (1978) Arch. Biochem. Biophys. 186, 416–421.
[17] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[18] Whatley, F. R. and Arnon, D. I. (1963) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N. O. eds) vol. 2, pp. 308–313, Academic Press, New York.
[19] McCarty, R. E. and Racker, E. (1967) J. Biol. Chem. 242, 3435–3439.
[20] Izawa, S. and Good, E. E. (1972) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N. O. eds) vol. 24 B, pp. 335–337, Academic Press, New York.
[21] Andreo, C. S. and Vallejos, R. H. (1973) FEBS Lett. 33, 201–204.
[22] Levy, H. M., Leher, P. D. and Ryan, E. M. (1963) J. Biol. Chem. 239, 3654–3659.
[23] Arana, J. L., Yoshida, M., Kagawa, Y. and Vallejos, R. H. (1980) submitted.
[24] Malyan, A. N. (1979) Dokl. Acad. Nauk SSSR 247, 993–996.
[25] Shoshan, V. and Selman, B. R. (1979) FEBS Lett. 107, 413–418.