TWO DIFFERENT TYPES OF ESSENTIAL CARBOXYL GROUPS IN CHLOROPLAST COUPLING FACTOR

Jose L. ARANA and Rubén H. VALLEJOS

Centro de Estudios Fotosintéticos y Bioquímicos (Consejo Nacional de Investigaciones Científicas y Técnicas, Fundación M. Lillo, Universidad Nacional de Rosario) Suipacha 531, 2000 Rosario, Argentina

Received 22 October 1980

1. Introduction

Photosynthetic formation of ATP in chloroplasts requires coupling factor 1 (CF_1) which is associated with the thylakoid membranes. This enzyme has been purified to homogeneity [1] and has a Me^{2+}-dependent, latent ATPase activity.

Studies of chemical modification of CF_1 with reagents specific for different amino acid residues such as arginine [2,3], lysine [4,5] and carboxyl groups [6,7] suggested that they may play a functional role in the catalytic mechanism or in the binding of substrates to the enzyme.

In [7] we showed that CF_1 was inactivated by Woodward's reagent K, a specific reagent for carboxyl groups which has been extensively used in chemical modification experiments [8–11]. The ATPase activities of both chloroplasts and soluble CF_1 were inactivated when one carboxyl group per active site was derivatized by Woodward's reagent K. The results in [7] suggested that the carboxyl group modified by Woodward's reagent K was different from that modified by dicyclohexylcarbodiimide (DCCD). In [6], inactivation of CF_1 by DCCD required 2 mol DCCD/mol CF_1 and the binding sites were found to be located in the β-subunit of CF_1.

Here, we present evidence that there are two different types of essential carboxyl groups in CF_1 which are selectively modified by either Woodward's reagent K or DCCD.

2. Experimental

CF_1 was purified from fresh market spinach leaves (*Spinacea oleracea* L.) and its latent ATPase activity was activated by heat and assayed as in [12].

Chemical modification of CF_1 (0.5 mg/ml) with Woodward's reagent K was at 25°C in 40 mM tricine–NaOH (pH 7.9). Aliquots were taken at different times and assayed for ATPase activity. Derivatization with DCCD was done similarly but at 30°C in 40 mM Mops–NaOH (pH 7.4).

Incorporation of [^{14}C]DCCD (160 μM) to CF_1 (1 mg/ml) was carried out essentially as performed with non-labelled DCCD. At different times the [^{14}C]-DCCD-bound ATPase was freed from unbound reagent by the elution–centrifugation method in [13]. Radioactivity of the samples was counted in a Beckman LS 233 liquid scintillation counter using 5 ml cocktail fluid (5 g 2,5-diphenyloxazole and 100 g naphtalene in 1 liter of 1,4 dioxane) and was corrected for background by omission of the enzyme in the reaction mixture. [^{14}C]ADP binding experiments were done as in [14] only at 30°C. For these experiments CF_1 was dialyzed against 100 vol. 40 mM tricine–NaOH, 2 mM EDTA pH 8 during 1 h and passed through Sephadex G-50 equilibrated with 10 mM MOPS–NaOH (pH 7). Treatment with Woodward's reagent K and DCCD was as above.

Protein was measured as in [15] with bovine serum albumin as standard.

[^{14}C]DCCD (50 mCi/mmol) was purchased from the Commissariat a l'Energie Atomique and [^{14}C]ADP (52.7 mCi/mmol) was obtained from New England Nuclear; Woodward's reagent K, ATP, ADP, were from Sigma. All other chemicals used were of analytical grade.

3. Results

Table 1 shows that ADP, ATP or P_i protected the ATPase activity of CF_1 against modification by Wood-

Table 1
Effect of different compounds on inactivation of ATPase activity of CF_1 by Woodward's reagent K and DCCD

Additions (mM)	$t_{0.5}$ (min)	
	WRK	DCCD
None	0.5	12
ATP (20)	1.3	13
ADP (20)	1.5	12
P_i (20)	1.1	13
$CaCl_2$ (5)	n.d.	20
$CaCl_2$ (20)	0.4	35
$CaCl_2$ (50)	n.d.	72
$CdCl_2$ (5)	n.d.	40
$CdCl_2$ (10)	n.d.	68
$CdCl_2$ (20)	0.5	85
$MgCl_2$ (20)	0.5	45
ATP (20), $CaCl_2$ (20)	1.4	20
ADP (20), $CaCl_2$ (20)	1.5	n.d.
ATP (5), $CdCl_2$ (5)	n.d.	24

Half-times of inactivation ($t_{0.5}$) of the ATPase activity of CF_1 by 1 mM Woodward's reagent K (WRK) or 160 μM DCCD are reported with the additions stated; n.d., not determined

ward's reagent K but not by DCCD. On the other hand divalent cations like Ca^{2+}, Cd^{2+} or Mg^{2+} afforded considerable protection of CF_1 against DCCD but not against Woodward's reagent K. Cd^{2+} was more effective as a protective agent than Ca^{2+} or Mg^{2+} since lower concentrations were required to achieve the same extent of protection. When both adenine nucleotides and cations were present during inactivation of CF_1 by DCCD less protection was obtained than by the cations alone probably because their actual concentrations were diminished by complexing with ADP or ATP.

The protection afforded by Ca^{2+} of CF_1 against inactivation by DCCD increased with pH (fig.1) while the protection given by Cd^{2+} or Mg^{2+} were less affected by pH in the range reported. It is noteworthy that inhibition of ATPase activity of CF_1 by DCCD diminished with higher pH [6]. Fig.2 shows that the rate of incorporation of [^{14}C]DCCD into CF_1 was diminished if Ca^{2+} or Cd^{2+} were present during modification. Although 10 mM $CdCl_2$ and 50 mM $CaCl_2$ afforded similar protection to CF_1 against DCCD (table 1) the latter was more effective in preventing incorporation of [^{14}C]DCCD into CF_1 (fig.2). This is more clearly seen in fig.3 were the amount of [^{14}C]DCCD incorporated into CF_1 was plotted against the percentage of inactivation of ATPase. These data confirmed

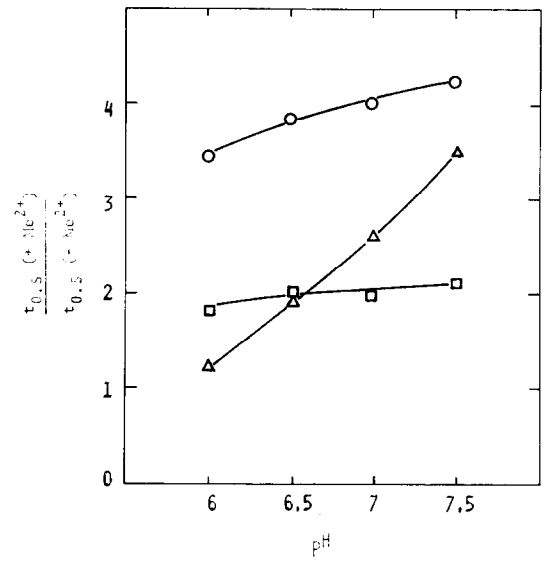

Fig.1. Effect of pH on the protection afforded by divalent cations to CF_1 against DCCD inactivation. Heat-activated CF_1 (0.5 mg/ml) was preincubated at 30°C with 160 μM DCCD in a reaction medium containing 20 mM Mes–NaOH, 20 mM Mops–NaOH and 20 mM tricine–NaOH at the stated pH in the absence or in the presence of (△) 20 mM $CaCl_2$, (○) 20 mM $MgCl_2$ or (□) 20 mM $CdCl_2$. Half-times of inactivation ($t_{0.5}$) of ATPase activity of CF_1 were determined in the absence or presence of Me^{2+} at different pH values.

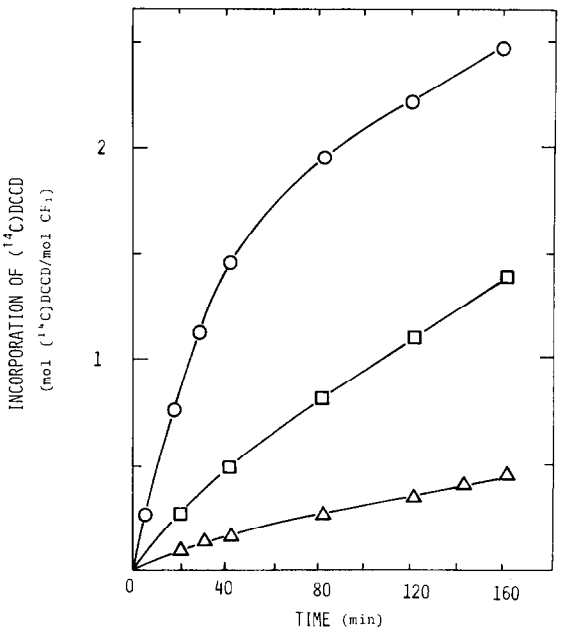

Fig.2. Time course of [^{14}C]DCCD incorporation into heat-activated CF_1. CF_1 was modified by [^{14}C]DCCD in the absence (○) or in the presence of 10 mM $CdCl_2$ (□) or 50 mM $CaCl_2$ (△).

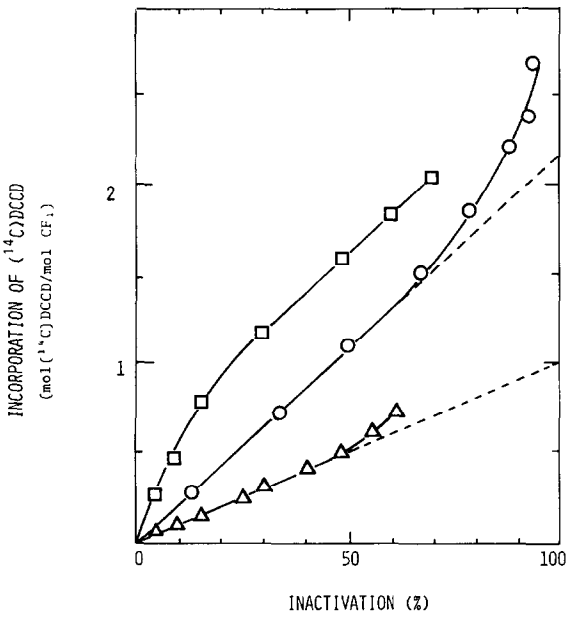

Fig.3. Correlation between inactivation of the ATPase activity and [^{14}C]DCCD incorporation into heat-activated CF_1. CF_1 was modified by [^{14}C]DCCD in the absence (○) or in the presence of 50 mM $CaCl_2$ (△) or 10 mM $CdCl_2$ (□). [^{14}C]DCCD incorporation and ATPase activity were measured as described in the text.

[6] that 2 mol [^{14}C]DCCD are bound/mol CF_1 when the ATPase was completely inactivated. This stoichiometry diminished to only 1 mol [^{14}C]DCCD bound/mol CF_1 when 50 mM $CaCl_2$ was present. $CdCl_2$ was less effective in preventing incorporation of DCCD than in affording protection against inactivation of the ATPase.

Table 2 shows that derivatization of CF_1 with Woodward's reagent K resulted in a 30% inactivation of the [^{14}C]ADP exchange in both latent and heat-activated CF_1. On the other hand, table 2 confirms [6] that the adenine nucleotide binding site exposed by heat treatment of CF_1 was completely inhibited by DCCD, while the pre-existing site in the latent ATPase remains unaffected [14].

4. Discussion

These results clearly suggest that there are two different types of functional carboxyl groups in CF_1. The evidence is as follows:

(i) One of them is modified by a hydrophylic reagent, i.e., Woodward's reagent K while the other one by a hydrophobic reagent; i.e. DCCD.

(ii) The protection afforded to each type of carboxyl groups by several compounds was different: the carboxyl groups modified by Woodward's reagent K were protected by adenine nucleotides and P_i while those modified by DCCD were protected only by divalent cations (table 1).

(iii) Modification by DCCD did not affect the ADP exchange reaction in native CF_1 while it completely supressed the new adenine nucleotide site that appears after heat activation [6]. On the other hand, Woodward's reagent K affected similarly all the adenine nucleotide sites (table 2).

(iv) Previous modification of CF_1 by 0.5 or 2 mM Woodward's reagent K did not prevent the incorporation of [^{14}C]DCCD in CF_1 in experiments similar to those of fig.2.

Table 2
Effect of Woodward's reagent K and DCCD on ATPase and ADP binding to CF_1

Expt.	Treatment of CF_1	[^{14}C]ADP binding (mol/mol CF_1)	Inactivation of ATPase activity (%)
1	None	0.44	0
	Modified by WRK	0.32	94
	Modified by DCCD	0.45	88
2	None	0.80	0
	Modified by WRK	0.58	96
	Modified by DCCD	0.42	90

Experiment 1 was performed with latent CF_1 and expt. 2 with the heat-activated enzyme. When stated CF_1 was treated with 2 mM Woodward's reagent K (WRK) or 160 μM DCCD and then [^{14}C]ADP binding and ATPase activity of CF_1 were measured as described in the text

Two different types of carboxyl groups have also been observed in mitochondrial F_1 since carboxyl reagents such *N*-cyclohexyl-*N'*-β(4 methylmorpholine) ethylcarbodiimide [17] and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline [18] did not interfere with the binding of DCCD [19].

Divalent cations afforded a strong protection against inactivation by DCCD. The effect of Ca^{2+} was markedly affected by pH, increasing with it (fig.1). This observation may be related to the fact that the ATPase activity of CF_1 with 5 mM $CaCl_2$ as cofactor increased 18 times between pH 6 and 8 while the activity with $MgCl_2$ and $CdCl_2$ increased only 9 and 5 times, respectively (not shown).

Ca^{2+} strongly prevented the incorporation of 1 mol labelled DCCD into CF_1 (fig.3) suggesting that modification of only 1 of the 2 carboxyl groups modified by DCCD is essential for activity. These results suggest that the carboxyl groups derivatized by DCCD play a role in binding the divalent cation to a functional site of CF_1. This suggestion is in agreement with the proposal [17] that a carboxyl group is a ligand of Me^{2+} in the active site of F_1. It is noteworthy that the coupling factors from beef heart mitochondria [19] and *E. coli* [20] are also inactivated by DCCD and protected by $MgCl_2$.

The protection afforded to CF_1 by ADP and ATP against inactivation by Woodward's reagent K suggests that the modified carboxyl groups may play a role in the adenine nucleotide binding sites although there is no direct evidence for it. The finding of a direct involvement of a carboxyl group in the pyridine nucleotide binding site of ferredoxin-NADP reductase shown by modification with the same reagent [21] may be relevant to this suggestion.

Acknowledgements

This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina) and the project PNUD/UNESCO RLA 78/024. R. H. V. is a Career Investigator and J. L. A. a Fellow of the former institution.

References

[1] Farron, F. (1970) Biochemistry 9, 3823–3828.
[2] Andreo, C. S. and Vallejos, R. H. (1977) FEBS Lett. 78, 207–210.
[3] Vallejos, R. H., Viale, A. M. and Andreo, C. S. (1977) FEBS Lett. 84, 304–308.
[4] Sugiyama, Y. and Mukohata, Y. (1978) FEBS Lett. 85, 211–214.
[5] Sugiyama, Y. and Mukohata, Y. (1979) FEBS Lett. 98, 276–280.
[6] Shoshan, V. and Selman, B. R. (1980) J. Biol. Chem. 255, 384–389.
[7] Arana, J. L. and Vallejos, R. H. (1980) FEBS Lett. 113, 319–322.
[8] Petra, P. H. (1971) Biochemistry 10, 3167–3170.
[9] Brake, A. J. and Weber, B. H. (1974) J. Biol. Chem. 249, 5452–5457.
[10] Dunn, B. M. and Anfinsen, C. B. (1974) J. Biol. Chem. 249, 3717–3723.
[11] Saini, M. S. and Vanetten, R. L. (1979) Biochim. Biophys. Acta 568, 370–376.
[12] Andreo, C. S., Ravizzini, R. A. and Vallejos, R. H. (1979) Biochim. Biophys. Acta 547, 370–379.
[13] Penefsky, H. S. (1977) J. Biol. Chem. 252, 2891–2899.
[14] Shoshan, Y. and Selman, B. R. (1979) FEBS Lett. 107, 413–417.
[15] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. L. (1951) J. Biol. Chem. 193, 265–275.
[16] Cantley, L. C. and Hammes, G. G. (1975) Biochemistry 14, 2968–2975.
[17] Koslov, I. A. and Skulachev, V. P. (1977) Biochim. Biophys. Acta 463, 29–89.
[18] Pougeois, R., Sastre, M. and Vignais, P. V. (1978) Biochemistry 17, 3018–3023.
[19] Pougeois, R., Sastre, M. and Vignais, P. V. (1979) Biochemistry 8, 1408–1412.
[20] Sastre, M., Lunardi, J., Pougeois, R. and Vignais, P. V. (1979) Biochemistry 18, 3134–3140.
[21] Carrillo, N., Arana, J. L. and Vallejos, R. H. (1981) Proc. Vth Intl. Congr. Photosynth. in press.