MODIFICATION OF ONE LYSINE BY PYRIDOXAL PHOSPHATE COMPLETELY INACTIVATES CHLOROPLAST COUPLING FACTOR 1 ATPase

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1. Introduction

Chemical modification of chloroplast coupling factor 1 (CF_1) in situ caused energy-transfer inhibition [1–3] of photophosphorylation and revealed essential arginyl [1,4] and lysyl [2,3] residues.

When CF_1 in situ was modified by pyridoxal phosphate (PLP) [3], the α, β and γ subunits were equally labeled and phosphorylation was inhibited. For 50% inhibition, 1 mol PLP was found in each of these 3 subunit fractions per mol CF_1. Since CF_1 has an allotopic nature [5], it was of interest to examine PLP modification on isolated CF_1, which was thus isolated from thylakoids, modified by PLP, then assayed for the heat-activated Ca^{2+}-ATPase [6].

It was found that modification of only 1 lysyl residue/CF_1 inactivated the Ca^{2+}-ATPase completely. For 50% inhibition, 0.5 mol PLP was found in each of the α and β subunit fractions per mol CF_1, but none in the γ subunit fraction.

2. Materials and methods

CF_1 was isolated from spinach chloroplasts by the method in [7] except that the density gradient centrifugation was omitted.

CF_1 (200–300 μg) was mixed in a medium containing 20 mM tricine–NaOH (pH 8.0), 1 mM EDTA, 10 mM $MgCl_2$ and a given concentration of PLP, incubated for a given time at 15°C in the dark, then modified by reducing the Schiff base complex with $NaBH_4$ [8]. PLP was thus covalently bonded to CF_1.

The CF_1 after PLP modification was passed through a column (1 × 13 cm) of Sephadex G-25, then placed in a medium containing 20 mM tricine–NaOH (pH 8.0), 1 mM EDTA, 25 mM ATP and 2 mM dithiothreitol and incubated at 62°C for 2.5 min to activate the latent ATPase [6,9]. The Ca^{2+}-ATPase was assayed for liberated P_i at 37°C and pH 8.5 by the method in [10]. Protein was assayed as in [11].

For the total amounts of bonded PLP per CF_1 (mol. wt 325 000 [12]), PLP was determined either by A_{325}, using ϵ 1 × 10^4 $M^{-1}.cm^{-1}$ for N^{ϵ}-phosphopyridoxyl lysine [13], or by the radioactivity of [^{3}H]PLP which was synthesized by the method in [14] and bonded to CF_1 as above. For the distribution of PLP among subunits of CF_1, [^{3}H]PLP-bonded CF_1 was electrophoresed on a polyacrylamide disc gel (10%) in the presence of sodium dodecyl sulfate (SDS) (0.1%). The radioactivity on the gel slices was compared with the stained pattern of the subunits as in [3].

3. Results and discussion

Figure 1 shows the incubation-time dependence of the amounts of bound PLP and the extent of Ca^{2+}-ATPase inactivation. Both saturate after incubation for >30 min, depending on the concentration of PLP in the modification mixture.

After the incubation of CF_1 with 80 μM PLP for 2 h, 1.2 mol PLP were bound per mol CF_1. When the same modification mixture was diluted 10-times with the buffer before the $NaBH_4$ reduction, the amounts of bound PLP decreased after dilution, following first order kinetics and after 1 h 0.1 mol PLP/CF_1

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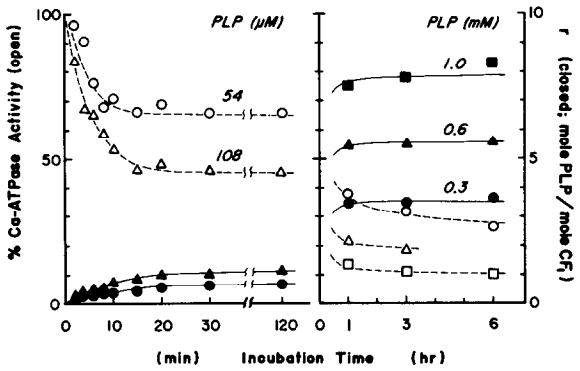

Fig.1. Incubation-time dependence of the Ca^{2+}-ATPase activity and the mole ratio of bonded PLP to CF_1 at varying concentrations of PLP. CF_1 was incubated for a given time at a given PLP concentration at 15°C in the dark then fixed with $NaBH_4$, and assayed for the ratio, r, or for Ca^{2+}-ATPase after heat activation. The control ATPase ([PLP] = 0) hydrolyzed ATP at a rate of 12.5 μmol/mg CF_1, min.

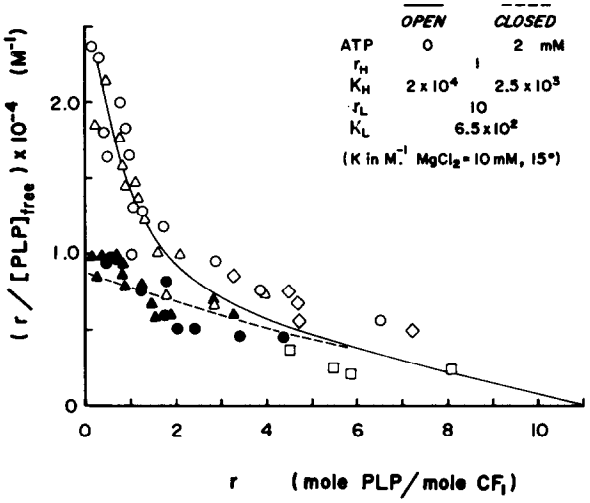

Fig.2. Scatchard plots for the binding of PLP to CF_1. Different marks represent different experiments. The binding was tested in the presence (closed marks) or absence (open marks) of 2 mM ATP. The curves were calculated with the values listed in the figure. For notation, see text.

was found. This implies that the Schiff base formation between CF_1 and PLP is reversible unless the base is reduced. When $NaBH_4$ was decomposed by trichloroacetic acid (TCA) (5%) at either 5 s or 5 min after the addition of $NaBH_4$ to the modification mixture that had been incubated for 1 h, the equal amounts of PLP were found in both CF_1 samples. These results indicate that the rate of PLP–CF_1 bond formation is sufficiently fast (cf. equilibrium shift rate), so that the amount of bound PLP represents the PLP–CF_1 complex (Schiff base) present at the instance of $NaBH_4$ addition.

When ATP or ADP was present in the modification mixture the Ca^{2+}-ATPase activity was preserved in the CF_1 after modification (see fig.3). This suggests that PLP binds close to or at the binding site(s) for adenine nucleotides, or that these nucleotides are effectors for a conformation change of CF_1 which masks the PLP binding site.

The amounts of bonded PLP per CF_1 were measured in the presence or absence of 2 mM ATP and analyzed in Scatchard plots [15] assuming binding equilibrium. The plots shown in fig.2 suggest the presence of >10 binding sites, differing in affinity.

Assuming 2 independent groups of PLP binding sites, the ratio (r), mol bound PLP/mol CF_1, is given by [16]:

$$r = \frac{r_H \quad K_H\,[PLP]}{1 \quad + K_H\,[PLP]} + \frac{r_L \quad K_L\,[PLP]}{1 \quad + K_L\,[PLP]}$$

where, [PLP] is the concentration of free PLP, K is a binding constant (in M^{-1}) of the ligand and the subscripts H and L denote the higher and lower affinity sites, respectively. The solid line in fig.2 was calculated with $r_H = 1$ at $K_H = 2 \times 10^4$ and $r_L = 10$ at $K_L = 6.5 \times 10^2$. The dashed line was calculated as above except for $K_H = 2.5 \times 10^3$ in the presence of ATP. The binding constant for ATP was also estimated to be $K_{ATP} = 3.5 \times 10^3$ under the assumption that ATP competes with PLP at the high affinity site. It should be noted that in the absence of $MgCl_2$, PLP bound to the sites only with very low affinity and caused little inactivation of Ca^{2+}-ATPase [17].

Figure 3 shows the amounts of bound PLP and the ATPase activity against the PLP concentration in the modification mixture (after 1 h incubation). When isolated CF_1 was first activated for Ca^{2+}-ATPase activity, then modified by PLP, the profiles were almost identical to those in fig.3. Thus PLP binds to the specific site(s) on the protein regardless of the ATPase being latent or active.

In the absence of ATP, Ca^{2+}-ATPase inactivation

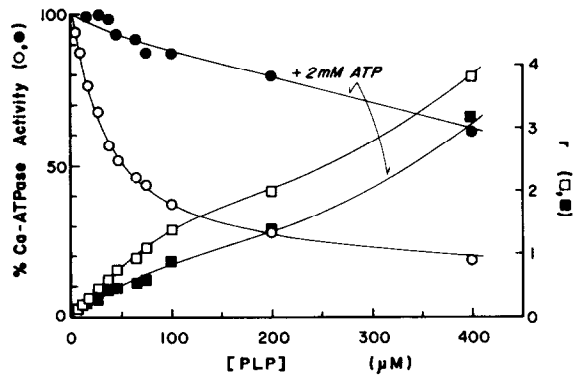

Fig.3. Remaining Ca^{2+}-ATPase activity and the mole ratio of bonded PLP to CF_1 after modification with varying concentrations of PLP in the presence or absence of ATP. CF_1 was incubated for 1 h with a given concentration of PLP with (closed marks) or without (open marks) 2 mM ATP, fixed with $NaBH_4$ and determined the ratio, r (in mol bonded PLP/mol CF_1) or assayed for ATPase after heat activation.

increased with the increase of bound PLP, and the activity was halved at 60 μM PLP (fig.3). Extrapolation of the plots of remaining ATPase activity against the bound PLP/CF_1 showed that about 1.5 mol PLP/CF_1 is required for complete inactivation (fig.4A;

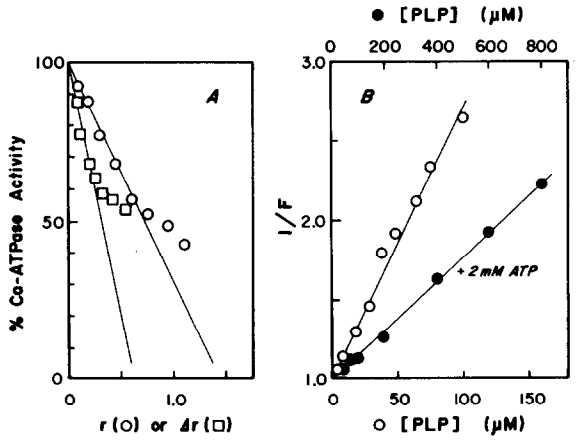

Fig.4. Replots of the data in fig.3. (A) The remaining Ca^{2+}-ATPase activity against the mole ratio, r, of bonded PLP to CF_1 (circles) and the differences due to 2 mM ATP in ATPase activity against those in the ratio, Δr (squares). (B) Reciprocals of the fractional activity, F, of Ca^{2+}-ATPase after modification with varying concentration of PLP in the presence (closed circles) or absence (open circles) of 2 mM ATP. For F, see text.

circles). In the presence of ATP, the ATPase was protected from inactivation but the amounts of bound PLP did not markedly decrease. The differences due to ATP in the remaining activity and in the amounts of bound PLP/CF_1 (Δr) were plotted in fig.4A (squares) and extrapolated to give a ratio of <1 for complete inactivation.

If multiple sites were modified simultaneously with the specific active site(s), the former extrapolation would give an overestimated ratio and the latter an underestimated one. Therefore, it is likely that modification of only 1 site on isolated CF_1 by PLP results in complete inactivation of Ca^{2+}-ATPase.

If this 1 (active) site binds either PLP or ATP, neglecting other non-specific sites, in the presence of both PLP and ATP at an equilibrium,

$$[E] + [PLP] = [E{-}PLP], \quad K'_{app} = \frac{[E{-}PLP]}{[E]\ [PLP]}$$

$$[E] + [ATP] = [E{-}ATP], \quad K'_{ATP} = \frac{[E{-}ATP]}{[E]\ [ATP]}$$

where, [E], [PLP], [E–PLP], [ATP] and [E–ATP] are concentrations of free ATPase, free PLP, PLP-bound ATPase, free ATP and ATP-bound ATPase, respectively. K'_{app} and K'_{ATP} are apparent binding constants (in M^{-1}, henceforth) for PLP and ATP to the active site, respectively. Since the E–PLP is inactive, the fractional remaining activity, F, is:

$$F = \frac{[E] + [E{-}ATP]}{[E] + [E{-}ATP] + [E{-}PLP]} = \frac{1 + K'_{ATP}\,[ATP]}{1 + K'_{ATP}\,[ATP] + K'_{app}\,[PLP]}$$

then,

$$\frac{1}{F} = 1 + \frac{K'_{app}\,[PLP]}{1 + K'_{ATP}\,[ATP]} = 1 + K''_{app}\,[PLP]$$

therefore, plots of $1/F$ versus [PLP] should be linear at $[E]_{total} \ll [PLP]$. The data in fig.3 were replotted in fig.4B to find this linear relationship from which

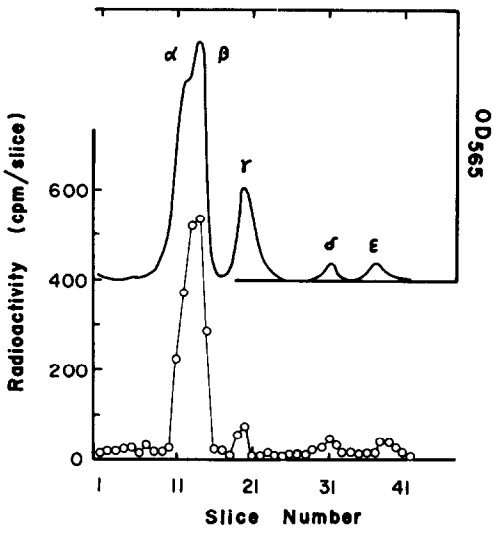

Fig.5. Labelling of CF_1 subunits by [^{3}H]PLP. CF_1 was incubated with 120 μM [^{3}H]PLP for 1 h in the dark and fixed with $NaBH_4$. Polyacrylamide gel (10%) electrophoresis was carried out with the CF_1 solution in the presence of SDS (0.1%). The gel was stained, sliced, digested by H_2O_2 and determined for radioactivity as in (3). The Ca^{2+}-ATPase activity of this CF_1 sample was found to be 35% of the control (without modification) and the ratio of bonded PLP/CF_1 was 1.5.

were estimated $K''_{app} = K'_{app} = 1.7 \times 10^4$ in the absence of ATP, and $K''_{app} = 1.5 \times 10^3$, then $K'_{ATP} = 5 \times 10^3$ in the presence of ATP. These values from the activity data (K'_{app} and K'_{ATP}) are close to the corresponding K values from the binding data (K_H and K_{ATP}).

These results support the assumptions that have been made and suggest that the Ca^{2+}-ATPase activity is completely lost by PLP modification of only 1 site, most probably a lysyl residue [3], which has the highest affinity to PLP.

Figure 5 shows the distribution of [^{3}H]PLP among the subunits of CF_1 separated on SDS–polyacrylamide disc gels [3]. Radioactivity was found in the α and β subunits to a much larger extent than in the γ subunit. When Ca^{2+}-ATPase activity was halved, about 0.5 mol PLP was found in each of the α and β subunit fractions per mol CF_1. Which of these subunits carries the active site has not been assigned yet.

The membrane-bound CF_1 was modified by PLP with its α, β and γ subunits and lost its catalytic activity [3]. The absence of PLP from the γ subunit of isolated CF_1 seems to be a reflection of the allotopic nature of CF_1 [5]. This may be related to the lack of inhibition of Ca^{2+}-ATPase of isolated CF_1 by orange I which inhibits photo- and acid–base-phosphorylation and light–thiol-activated Mg^{2+}-ATPase in chloroplasts in competition with nucleotide substrates (Y.M. et al. in preparation).

Since there are at least pairs of α and β subunits [18] in 1 CF_1, the complete inactivation of Ca^{2+}-ATPase by modification of a single PLP/CF_1 would require a sophisticated mechanism for ATPase action (see [17]).

Present results suggest possible changes in the intra- and/or inter-subunit architecture of CF_1 when CF_1 is detached from the thylakoid, and the presence of 1 essential lysyl residue for Ca^{2+}-ATPase in either the α or the β subunit per mol isolated CF_1.

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References

[1] Schmid, R., Jagendorf, A. T. and Hulkower, S. (1977) Biochim. Biophys. Acta 462, 177–186.
[2] Schopf, R. and Harnischfeger, G. (1977) Z. Naturforsch. 32c, 398–404.
[3] Sugiyama, Y. and Mukohata, Y. (1978) FEBS Lett. 85, 211–214.
[4] Vallejos, R. H., Viala, A. and Andreo, C. S. (1977) FEBS Lett. 84, 304–309.
[5] Racker, E. (1976) A New Look at Mechanisms in Bioenergetics, Academic Press, New York.
[6] Vambutas, V. K. and Racker, E. (1965) J. Biol. Chem. 240, 2660–2667.
[7] Lien, S. and Racker, E. (1971) Methods Enzymol. 23, 547–555.
[8] Fischer, E. H., Kent, A. B., Synder, E. R. and Krebs, E. G. (1958) J. Am. Chem. Soc. 80, 2906–2907.
[9] Farron, F. and Racker, E. (1970) Biochemistry 9, 3829–3836.
[10] Taussky, H. and Shorr, E. (1953) J. Biol. Chem. 202, 675–685.
[11] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. L. (1951) J. Biol. Chem. 193, 265–275.
[12] Farron, F. (1970) Biochemistry 9, 3823–3828.

[13] Forry, A. W., Olsgaard, R. B., Nolan, C. and Fischer, E. H. (1971) Biochimie 53, 269–281.
[14] Stock, A., Ortanderl, F. and Pfleidere, G. (1966) Biochem. Z. 344, 353–360.
[15] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672.
[16] Edsall, J. T. and Wyman, J. (1958) Biophysical Chemistry, vol. 1. pp. 591–662, Academic Press, New York.
[17] Sugiyama, Y. and Mukohata, Y. (1979) in: Cation Flux across Biomembranes (Mukohata, Y. and Packer, L. eds) Academic Press, San Francisco, in press.
[18] Nelson, N., Kamienietzky, A., Deters, D. W. and Nelson, N. (1975) in: Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E. et al. eds) pp. 149–154, North-Holland, Amsterdam.