ENERGY TRANSFER INHIBITION INDUCED BY MODIFICATION OF MEMBRANE-BOUND CHLOROPLAST COUPLING FACTOR 1 BY PYRIDOXAL PHOSPHATE

Yasuo SUGIYAMA and Yasuo MUKOHATA

Department of Biology, Faculty of Science, Osaka University, Toyonaka, 560 Japan

Received 3 November 1977

1. Introduction

The function of the five different subunits [1] of chloroplast coupling factor 1 (CF_1) has been under active investigation. The modification of isolated CF_1 by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole [2] suggests the involvement of tyrosyl residues in the active sites on the β-subunits. The modifications by butane-2,3-dione [3,4] and fluorescamine [5] suggest the presence of essential arginyl and lysyl residue, respectively.

Recently, pyridoxal phosphate (PLP) was used to detect active site lysyl residues in various proteins [6–8] which interact with phosphate-containing ligands. In this paper, we report that the modification of membrane-bound CF_1 by PLP inhibits phosphorylation without damaging electron transport, suggesting the participation of lysyl residue(s) in the activity of CF_1.

2. Materials and methods

Chloroplasts were prepared from market spinach leaves in a choline medium by a method described [9]. Chlorophyll was determined by the method [10]. Chloroplasts equivalent to 400 μg chlorophyll/ml in 0.1 M sucrose, 10 mM tricine–NaOH (pH 8.3), and 5 mM $MgCl_2$ were incubated with PLP at 15°C for a given time in the dark. Reactions were stopped by adding $NaBH_4$ ([11]; 5–10 times in excess of the PLP concentration used). The modified chloroplasts were centrifuged at 4000 × *g* for 10 min then resuspended in the sucrose medium.

^{3}H-Labeled PLP was prepared according to the method in [12] withh slight modification. Specific radioactivity of [^{3}H]PLP was 5.1×10^7 cpm/μmol. Crude CF_1 was isolated by the chloroform extraction method [13] except that the Sephadex G-200 step was omitted. The subunits of CF_1 were separated on polyacrylamide (10%) disc gels in the presence of sodium dodecyl sulfate [14]. Gels were stained with Coomassie blue, destained in a mixture of methanol, acetic acid and water (2:3:20) and sliced. Each slice (1 mm thick) was put into a vial, digested by 0.5 ml H_2O_2 (30%), followed by the addition of 10 ml modified (10% Triton X-100 added) Bray's cocktail [15] and the radioactivity measured.

Electron transport (ferricyanide reduction) and phosphorylation were assayed by the methods described [9]. Light and dithiothreitol (DTT)-activated Mg^{2+}-ATPase and trypsin-activated Ca^{2+}-ATPase were assayed according to the method [16]. Protein was determined according to [17] with bovine serum albumin as standard.

3. Results and discussion

Figure 1 shows that incubation of chloroplasts with PLP for 10 min in the dark had negligible effect on electron transport in the presence of ATP and P_i but decreased it under phosphorylation conditions (ADP + P_i) in parallel with a decrease in phosphorylation. Similar results were reported for butanedione

Abbreviations: CF_1, chloroplast coupling factor 1; PLP pyridoxal phosphate; Fecy, ferricyanide; DTT, dithiothreitol

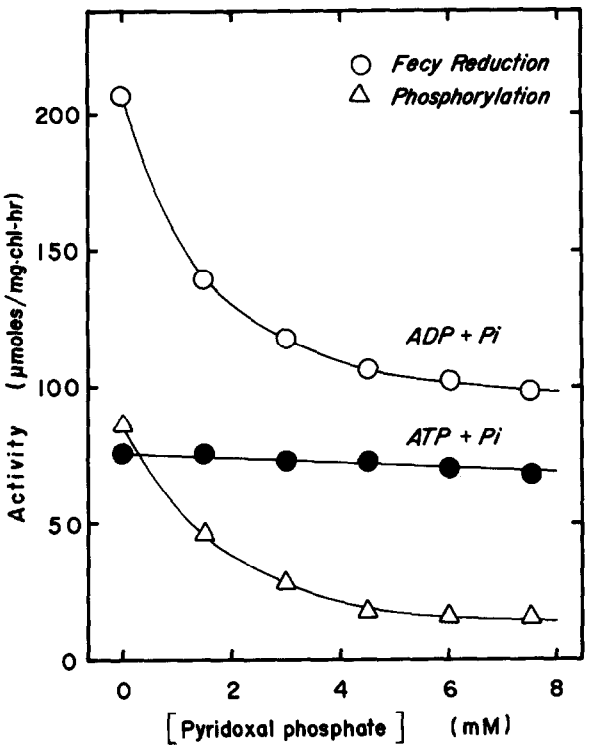

Fig.1. Effect of LPL modification on the activities of Fecy reduction and phosphorylation. Spinach chloroplasts were incubated with the indicated concentrations of PLP for 10 min in the dark, and the Schiff base of PLP was fixed by reduction with $NaBH_4$ as described in section 2. Fecy reduction was measured in the presence of 100 μM ATP and 1 mM P_i (•), or 500 μM ADP and 1 mM P_i (○). Phosphorylation coupled with Fecy reduction was measured in the presence of 500 μM ADP and 1 mM [^{32}P]P_i (△).

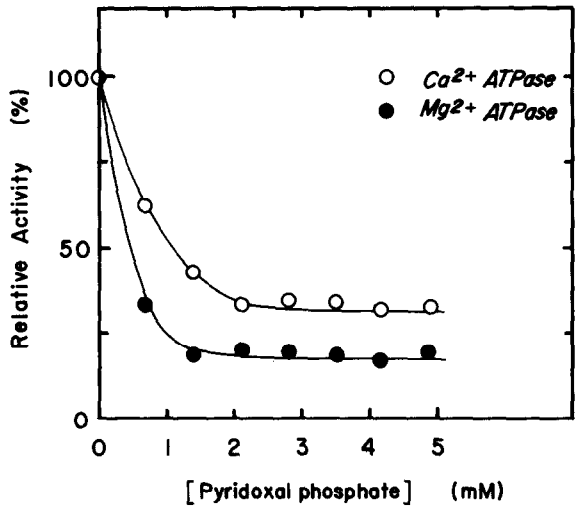

Fig.2. Effect of PLP modification on the activities of trypsin-activated Ca^{2+}-ATPase and light and DTT-activated Mg^{2+}-ATPase. Preincubation of chloroplasts with PLP was carried out as in fig.1. The rates of Ca^{2+}-ATPase and Mg^{2+}-ATPase of the control (100%) were 303 and 481 μmol P_i/mg · chl-h, respectively.

modification [4]. The ratio of the phosphorylation to coupled electron transport (i.e., electron transport (ADP + P_i) minus basal electron transport (ATP + P_i) [18] is constant in agreement with the results obtained with phlorizin [19] and dicyclohexylcarbodiimide [20]. Light-induced proton uptake was almost unchanged after PLP modification (data not shown), implying again that electron transport (and the resultant proton pump) were not damaged by the modification. These results indicate that PLP modification causes energy transfer inhibition. Addition of ADP and ATP to the incubation mixture retarded the inactivation of phosphorylation by PLP. When chloroplasts were incubated in light (5×10^4 lux, white light), uncoupling also occurred with an increase in basal electron transport activity [21].

Figure 2 shows that the light and DTT-activated Mg^{2+}-ATPase and the trypsin-activated Ca^{2+}-ATPase of membrane-bound CF_1 were inhibited after PLP modification. The results of fig.1 and 2 indicate that PLP modified the membrane-bound CF_1 itself in the dark and consequently, ATP hydrolysis or phosphorylation and the coupled electron transport were inhibited. In contrast with the modification of membrane-bound CF_1 by *N*-ethylmaleimide [22] which requires energization of the thylakoid membrane, the PLP modification inactivated phosphorylation in the dark. Thus, PLP can readily reach sites essential to phosphorylation without any energy-dependent change [23] in CF_1 conformation.

Figure 3A gives semilogarithmic plots of the remaining activity of phosphorylation versus incubation time showing that the process of phosphorylation inactivation followed pseudo-first order kinetics. Figure 3B shows that a slope of about 1 obtained in the log–log plot of the rate constant obtained from fig.3A versus PLP concentration. This suggests that the inactivation of phosphorylation by PLP is caused

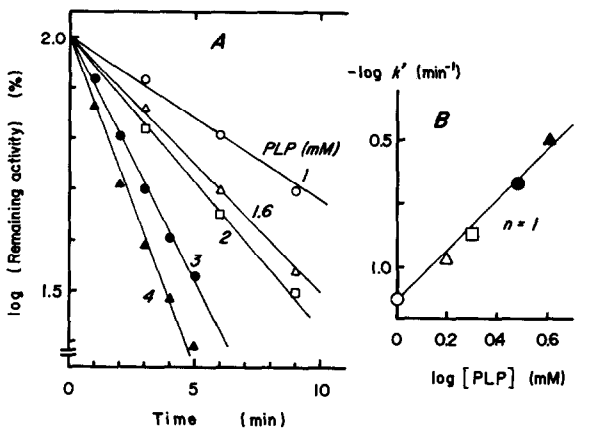

Fig.3. Analysis of the PLP modification kinetics of chloroplasts. (A) Semilogarithmic plots of the remaining activity of phenazine methosulfate-mediated phosphorylation versus incubation time of chloroplasts with PLP. The rate of phosphorylation of the control (100%) was 180 μmol ATP/mg · chl-h. (B) Log-log plots of the rate constants (k') obtained from fig.3A versus PLP concentration. The slope n indicates the reaction order.

by 1:1 interaction between the modifier and the active site. The kinetics are similar to those of butanedione modification of isolated (CF_1) ATPase [3].

Figure 4A shows the rate of Fecy reduction versus

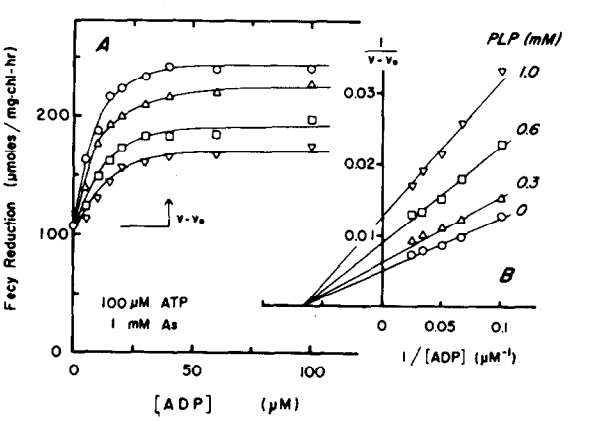

Fig.4. (A) Effect of PLP modification on the arsenylation-coupled stimulation of Fecy reduction. Chloroplasts were incubated for 10 min in the dark with 0.3, 0.6 or 1 mM PLP and fixed with $NaBH_4$. (B) Double reciprocal plots of arsenylation-coupled electron transport activity ($v-v_0$) versus ADP concentration. v_0 is the activity of Fecy reduction in the presence of 100 μM ATP and 1 mM arsenate.

ADP concentration under arsenylation conditions (ADP + arsenate) in chloroplasts partially modified by PLP (controlled by the PLP concentration). Double reciprocal plots of the arsenylation-coupled electron transport activity ($v-v_0$, where v_0 = the basal electron transport level [18]) versus ADP concentration (fig.4B) reveal that in PLP-modified chloroplasts the K_m value for ADP is the same as that of the control but the V_{max} value is lowered. The result is similar to that reported for Ca^{2+}-ATPase after butanedione modification [3] but different from the non-specific modification by 2,4,6-trinitrobenzenesulfonate [24] in which changes in both K_m and V_{max} were reported.

Figure 5 shows the distribution of radioactivity among subunit proteins of CF_1 separated on polyacrylamide gel in the presence of sodium dodecylsulfate by electrophoresis. CF_1 was isolated from

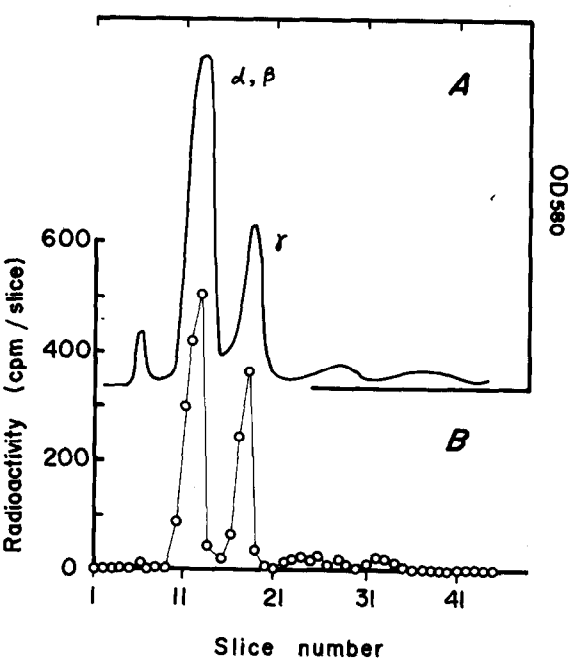

Fig.5. Labeling of CF_1 subunits by [^{3}H]PLP. CF_1 was isolated from chloroplasts which were incubated with 2 mM [^{3}H]PLP for 10 min in the dark and fixed with $NaBH_4$. A CF_1 solution (40 μl) containing 40 μg protein was applied to 10% polyacrylamide gel for electrophoresis. The densitometric trace of this crude CF_1 sample indicated contamination by proteins of low molecular weight. Subunits α and β ran together and the distribution of radioactivity between them could not be determined under this condition.

chloroplasts after [^{3}H]PLP modification which inhibited about 50% of the phosphorylation activity. About 3 mol PLP were bound/mol CF_1 in this experiment. By extrapolation, 6 ± 1 mol PLP bind to 1 mol CF_1 when phosphorylation is completely inactivated. The labeling pattern shows that the radioactivity was distributed mostly among the α, β and γ subunits with a ratio of $(\alpha + \beta) : \gamma = 2:1$ but apparently not the δ or the ϵ subunit. When the α and the β subunits were separated with a 12% gel, radioactivity was equally distributed among the α and the β subunits (not shown).

The PLP modification is thought to be due to a Schiff base formation with an ϵ-amino group of lysine [11]. When lysine was added at a final concentration of 40 mM before the $NaBH_4$ fixation, CF_1 was not modified by [^{3}H]PLP. The butanedione modification [3,4] suggested the presence of one essential arginine/active site. Our results sugggest the participation of one essential lysine per α and/or β and/or γ subunits. When chloroplasts were modified by [^{3}H]PLP after incubation with butanedione (e.g., at 100 mM), the number of labeled PLP on CF_1 decreased to almost one-third of the control (not shown). Thus, butanedione and PLP modify the same sites, and PLP further modifies non-essential sites which have not been identified.

Acknowledgements

We thank Dr A. T. Jagendorf of Cornell University and Dr M. Avron of Weizmann Institute for their stimulating discussion and helpful suggestions in preparing the manuscript.

References

[1] Lien, S., Berzborn, R. J. and Racker, E. (1972) J. Biol. Chem. 247, 3520–3524.
[2] Deters, D. W. and Racker, E. (1975) ibid. 250, 1041–1047.
[3] Andreo, C. S. and Vallejos, R. H. (1977) FEBS Lett. 78, 207–210.
[4] Schmid, R., Jagendorf, A. T. and Hulkower, S. (1977) Biochim. Biophys. Acta in press.
[5] Schopf, R. and Harnischfeger, G. (1977) Z. Naturforsch. 32c, 398–404.
[6] Colombo, G. and Murcus, F. (1974) Biochemistry 13, 3085–3091.
[7] Milhausen, M. and Levy, H. R. (1975) Eur. J. Biochem. 50, 453–461.
[8] Murphy, A. J. (1977) Arch. Biochem. Biophys. 180, 114–120.
[9] Mukohata, Y., Yagi, T., Matsuno, A., Higashida, M. and Sugiyama, Y. (1974) Plant Cell Physiol. 15, 163–167.
[10] Arnon, D. I. (1948) Plant Physiol. 24, 1–15.
[11] Fisher, E. H., Kent, A. B., Snyder, E. R. and Krebs, E. G. (1958) J. Am. Chem. Soc. 80, 2906–2907.
[12] Stock, A., Ortanderl, F. and Pfleiderer, G. (1966) Biochem. Z. 344, 353–360.
[13] Younis, H. M., Winget, G. D. and Racker, E. (1977) J. Biol. Chem. 252, 1814–1818.
[14] Weber, K. and Osborn, M. (1969) ibid. 244, 4406–4412.
[15] Bray, G. A. (1960) Anal. Biochem. 1, 279–285.
[16] Datta, D. B., Ryrie, I. J. and Jagendorf, A. T. (1974) J. Biol. Chem. 249, 4404–4411.
[17] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. L. (1951) ibid. 193, 265–275.
[18] Mukohata, Y., Yagi, T., Sugiyama, Y., Matsuno, A. and Higashida, M. (1975) Bioenergetics 7, 91–102.
[19] Izawa, S., Winget, G. D. and Good, N. E. (1966) Biochem. Biophys. Res. Commun. 22, 223–226.
[20] Yagi, T. and Mukohata, Y. (1977) J. Bioenerg. Biomembr. 9, 31–40.
[21] Mukohata, Y., Yagi, T. and Sugiyama, Y. (1975) Bioenergetics 7, 103–109.
[22] McCarty, R. E., Pittman, P. R. and Tsuchiya, Y. (1972) J. Biol. Chem. 247, 3048–3051.
[23] Ryrie, I. J. and Jagendorf, A. T. (1971) ibid. 246, 3771–3774.
[24] Oliver, D. and Jagendorf, A. T. (1976) ibid. 251, 7168–7175.