SPECIFIC LABELLING OF A PROTEIN INVOLVED IN PHOSPHATE TRANSPORT OF CHLOROPLASTS BY PYRIDOXAL-5'-PHOSPHATE

Ulf-Ingo FLÜGGE and Hans W. HELDT

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Goethestraße 33, 8000 München 2, FRG

Received 13 July 1977

1. Introduction

The phosphate translocator located in the inner membrane of the chloroplast envelope is an important part of the overall reaction of CO_2 fixation, enabling, in exchange with inorganic phosphate, the export of fixed carbon in the form of triosephosphates and 3-phosphoglycerate from the chloroplasts [1]. In order to identify and characterize the membrane protein involved in this carrier activity, the protein must be specifically labelled, e.g., by the incorporation of inhibitors. We have reported recently [2] that the phosphate translocator is inhibited by *p*-(diazonium)-benzene sulfonic acid, a substance known to react mainly with tyrosine and histidine. The radioactively labelled inhibitor was incorporated into several proteins of the envelope membrane, most of it into a membrane protein with mol. wt 29 000. The attachment of the radioactive inhibitor into this polypeptide only was specifically decreased by substrates known to be transported by the phosphate translocator. The data indicated that a 29 000 dalton polypeptide contains binding sites essential for the function of the phosphate translocator. The present publication describes in alternative approach for the identification of the carrier protein employing the labelling with pyridoxal-5'-phosphate. This compound reacts with the ϵ-amino group of lysine forming a Schiff base, which can be irreversibly reduced with $NaBH_4$. Due to its negative charge and the highly hydrophilic nature, pyridoxal-5'-phosphate is a membrane impermeant agent, which has been frequently used as a surface probe of several membrane systems [3–6]. It is therefore specially suited to react with proteins exposed to the outer surface of the inner envelope membrane. The present results show that pyridoxal-5'-phosphate inhibits phosphate transport in chloroplasts and is also incorporated into a membrane polypeptide with mol. wt 29 000.

Abbreviation: PLP, pyridoxal-5'-phosphate

2. Materials and methods

The chloroplasts were prepared from fully-grown spinach leaves according to the method of Cockburn et al. [7] modified by Heldt and Sauer [8]. For the assay of chlorophyll see [9] and of protein see [10]. Phosphate transport was measured by silicone-layer filtering centrifugation [1].

For the labelling procedure intact chloroplasts equivalent to 3 mg chlorophyll were suspended in 7 ml medium containing 0.33 M sorbitol, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (Hepes), pH 7.6, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA. This suspension was treated with 0.5 mM pyridoxal-5'-phosphate for 20 min. A four-fold molar excess of NaB^3H_4 (30 mCi/mmol) then was added and incubation proceeded at 4°C for 10 min. The chloroplasts were washed three times with medium. In order to remove the envelope membranes by osmotic shock, 4 ml 10 mM *N*-trishydroxymethyl-methyl-glycine (Tricine) pH 7.8, and 4 mM $MgCl_2$ were added. After 1.5 min at 4°C, sucrose was added to a final concentration of 0.33 M. Purification of

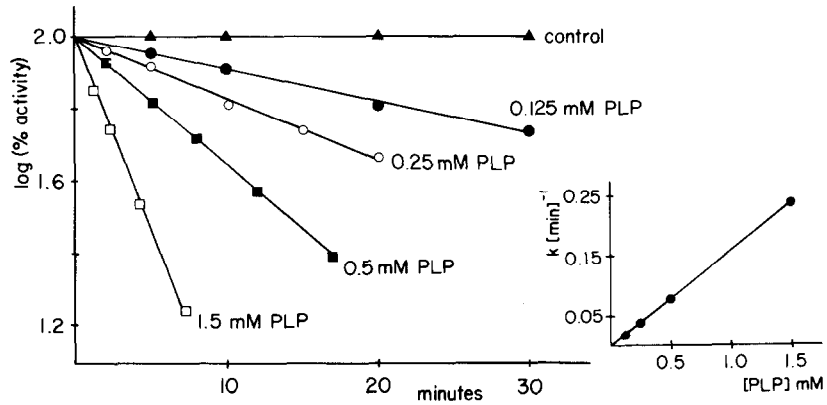

Fig.1. Time course of inhibition of phosphate transport by pyridoxal-5'-phosphate. The numbers beside the curves represent the varying concentrations of pyridoxal-5'-phosphate applied. Intact chloroplasts (0.1 mg/ml) were incubated with pyridoxal-5'-phosphate as indicated. At given times, incubations were stopped by silicone-layer filtering centrifugation. The uninhibited rate was 43 μmol/mg chlorophyll.h. Fig.1 inset. The pseudo first-order kinetics of the inhibition of phosphate transport with respect to pyridoxal-5'-phosphate. The rate constants (k) were obtained from the data shown in fig.1.

envelope membranes was achieved by discontinuous sucrose-gradient centrifugation as described earlier [2] according to the method of Douce et al. [11]. The purified envelope membranes were analyzed by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate [12]. The separation gel contained 11.5% acrylamide and 0.1% *N,N'*-methylene bisacrylamide. Tandem gels were run which were either stained with Coomassie Blue [13] and scanned at 546 nm or analyzed for radioactivity by slicing and digestion with NCS (Amersham and Searle) [14] followed by liquid scintillation counting.

All steps following the incubation with pyridoxal-5'-phosphate were done with minimal exposure to light.

3. Results and discussion

3.1. *Inactivation of phosphate transport by pyridoxal-5'-phosphate*

The inhibition of phosphate transport by pyridoxal-5'-phosphate is a time-dependent process, as shown in fig.1. Within the concentration range studied, the plots of the logarithm of transport activity versus the time of pyridoxal-5'-phosphate treatment are linear, indicating that the inactivation follows pseudo first-order kinetics with respect to remaining carrier activity.

The apparent first-order rate constant for inactivation calculated from data of fig.1 is directly proportional to the concentration of pyridoxal-5'-phosphate (fig.1 inset) indicating that inactivation is also first order with respect to pyridoxal phosphate and second order overall (k_2 160 $M^{-1}min^{-1}$, at pH 7.6 and 4°C). In fig.2 the logarithm of the reciprocal values of the half-time of inhibition ($t_{0.5}$) is plotted as a function

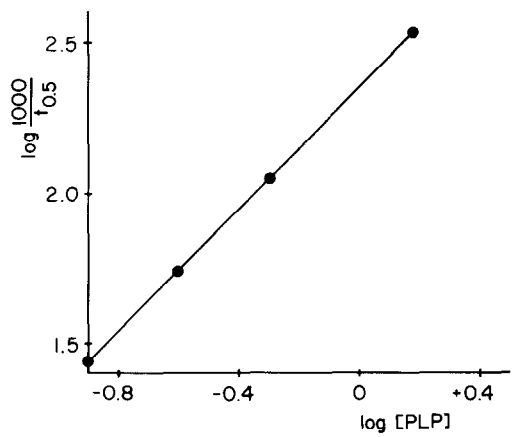

Fig.2. Determination of the order of the inactivation reaction with respect to pyridoxal-5'-phosphate. The half-time ($t_{0.5}$) values were obtained from the data of fig.1. The reciprocal of the log of the half-time was plotted against the log of pyridoxal-5'-phosphate concentration.

of the logarithm of the inhibitor concentration. A straight line is obtained with a slope equal to 1. According to previously published considerations [15,16] these data indicate that one inhibitor molecule is required per active site to produce an inactive carrier–inhibitor complex. The inhibition of phosphate transport by prolonged preincubation of the chloroplasts with the inhibitor can be partially overcome when the transport measurements are carried out with increased phosphate concentrations in the medium. The double reciprocal plot of the concentration dependence of the transport in the presence of the inhibitor shows the characteristics of a competitive inhibition, as shown in fig.3. In other experiments similar to those shown in fig.4 it was found that increasing phosphate concentration did not affect the time-dependent binding of the pyridoxal-5′-phosphate. This finding may be explained by the assumption that binding of the pyridoxal-5′-phosphate to the membrane protein via the formation of a Schiff base does not inhibit as such, but is a prerequisite that the phosphate group attached to the pyridoxal-5′-phosphate may be reversibly bound as a competitive inhibitor to the carrier protein.

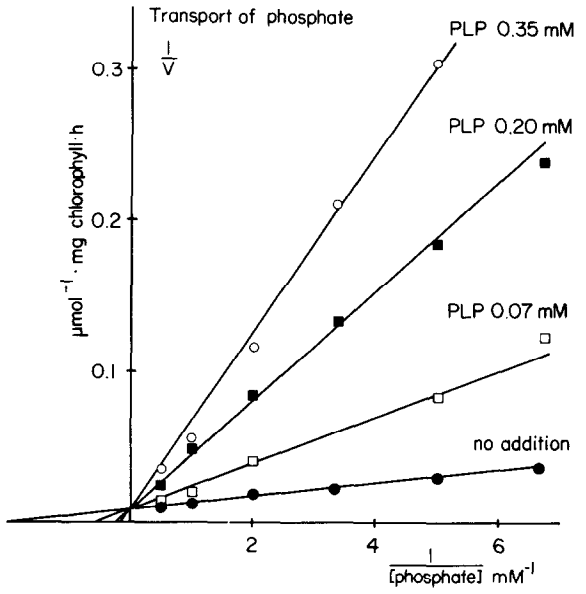

Fig.3. Phosphate translocator activity in the presence of pyridoxal-5′-phosphate as a function of phosphate concentration. Chloroplasts (0.1 mg/ml) were preincubated with different concentrations of pyridoxal-5′-phosphate as indicated in the figure for one hour. Phosphate transport was measured as described earlier [1].

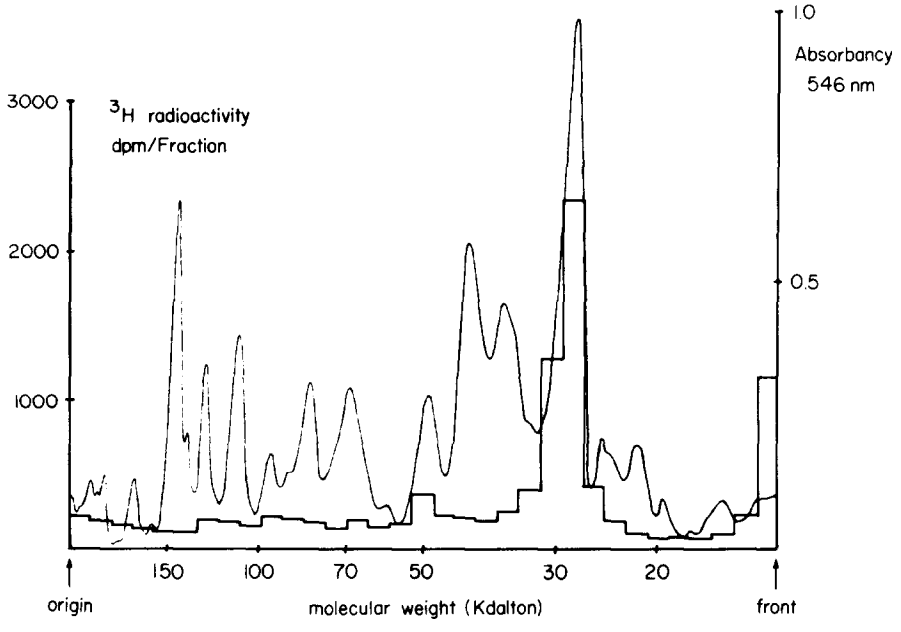

Fig.4. SDS–gel electrophoresis of envelope membrane proteins allowed to react with pyridoxal-5′-phosphate. Incubation of intact chloroplasts with pyridoxal-5′-phosphate, subsequent labelling by NaB^3H_4 and isolation of envelope membranes were carried out as described in methods. Membranes equivalent to 150 µg protein were applied to gel electrophoresis. Separate gels were used for the absorbance scan (continuous curve) and for radioactivity scan (discrete lines).

3.2. *Labelling of envelope membrane proteins by pyridoxal-5'-phosphate*

In the experiment of fig.4 the binding of pyridoxal-5'-phosphate to envelope membrane proteins was investigated. For this, the intact chloroplasts were incubated with pyridoxal-5'-phosphate. The pyridoxal-5'-phosphate bound to the protein was then radioactively labelled by incubation with NaB^3H_4, leading to an irreversible reduction of the Schiff base between the pyridoxal-5'-phosphate and the membrane proteins. Subsequently, the envelope membranes were isolated and the incorporation of the tritium label into membrane proteins was analyzed by SDS–gel electrophoresis. The resulting pattern shows that the radioactivity is almost exclusively appearing together with the major protein peak of mol. wt 29 000.

Because of the expected impermeability of the inner envelope membrane to pyridoxal-5'-phosphate, the labelled protein should be exposed to the outside of this membrane. When the inner envelope membrane is ruptured by osmotic shock prior to treatment with pyridoxal-5'-phosphate, other proteins of the envelope membrane, most probably accessible from the inside, become also labelled (traces not shown). It appears therefore, that the arrangement of the proteins in the envelope membrane is asymetric.

The question arises, whether the observed binding of pyridoxal-5'-phosphate to the membrane protein with mol. wt 29 000 as detected by the incorporation of the tritium label, correlates with the inhibition of phosphate transport. In order to answer this, chloroplasts were incubated with pyridoxal-5'-phosphate for various time intervals, and the inhibition of phosphate transport into intact chloroplasts and the incorporation of the tritium label into the 29 000 dalton protein of the envelope membrane were measured in parallel experiments. The data of fig.5 show that there is a linear relationship between these two parameters, indicating that the binding of pyridoxal-5'-phosphate to the 29 000 dalton polypeptide of the envelope membrane leads to an inhibition of phosphate transport. In agreement with our earlier findings [2] these data allow the conclusion, that a polypeptide with mol. wt 29 000 is involved in specific phosphate transport across the inner envelope membrane.

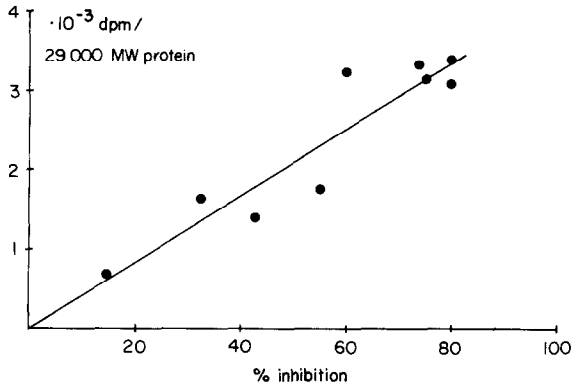

Fig.5. Correlation between the inhibition of phosphate transport and incorporation of pyridoxal-5'-phosphate into the 29 000 dalton protein. Chloroplasts (0.1 mg/ml) were incubated with 0.5 mM pyridoxal-5'-phosphate for various time intervals. In parallel experiments inhibition of phosphate transport and incorporation of pyridoxal-5'-phosphate into the 29 000 dalton protein were measured. Incubations were stopped either by silicon layer filtering centrifugation or by further incubation with NaB^3H_4. The envelope membrane proteins were isolated and analyzed for radioactivity as described in Materials and methods.

Acknowledgement

This research has been supported by the Deutsche Forschungsgemeinschaft. The financial support of U. I. Flügge by the Studienstiftung des Deutschen Volkes is gratefully acknowledged.

References

[1] Heldt, H. W. and Rapley, L. (1970) FEBS Lett. 10, 143–148.
[2] Flügge, U. I. and Heldt, H. W. (1976) FEBS Lett. 68, 259–262.
[3] Rifkin, D. B., Compans, R. W. and Reich, E. (1972) J. Biol. Chem. 247, 6432–6437.
[4] Cabantchik, I. Z., Balshin, M., Breuer, W. and Rothstein, A. (1975) J. Biol. Chem. 250, 5130–5136.
[5] Golds, E. E. and Braun, P. E. (1976) J. Biol. Chem. 251, 4729–4735.
[6] Bednarz-Prashad, A. J. and Mize, C. E. (1974) Biochemistry 13, 4237–4242.
[7] Cockburn, W., Walker, D. A. and Baldry, C. W. (1968) Biochem. J. 107, 89–95.

[8] Heldt, H. W. and Sauer, F. (1971) Biochem. Biophys. Acta 234, 83–91.
[9] Whatley, F. R. and Arnon, D. J. (1963) Method. Enzymol. 6, 308–313.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[11] Douce, R., Holtz, B. R. and Benson, A. A. (1973) J. Biol. Chem. 248, 7215–7222.
[12] Neville, D. M. (1971) J. Biol. Chem. 246, 6328–6334.
[13] Hoober, J. K. (1970) J. Biol. Chem. 245, 4327–4334.
[14] Basch, R. S. (1968) Analyt. Biochem. 26, 185–188.
[15] Marcus, F., Schuster, S. M. and Lardy, H. A. (1976) J. Biol. Chem. 251, 1775–1780.
[16] Scrutton, M. C. and Utter, M. F. (1965) J. Biol. Chem. 240, 3714–3723.