Volume 283, number 1, 57–60 FEBS 09734 May 1991

ADONIS 001457939100443V

Functional size analysis of pyrophosphatase from *Rhodospirillum rubrum* determined by radiation inactivation

Jia Jiu Wu, Jing Tyan Ma and Rong Long Pan

Institute of Radiation Biology and Nuclear Science, College of Nuclear Science, National Tsing Hua University, Hsinchu 30043, Taiwan, Republic of China

Received 8 February 1991; revised version received 19 March 1991

A radiation inactivation technique was employed to determine the functional size of pyrophosphatase (PPase) from the chromatophores of *Rhodospirillum rubrum*. The activities of hydrolysis and synthesis reactions of pyrophosphatase and its coupled proton translocation decayed in a simple exponential function with the increase of radiation dosages. D_{37} values of 5.2 ± 0.7 and 5.8 ± 0.8 Mrads were obtained for pyrophosphate hydrolysis and its associated proton translocation yielding molecular masses of 167.7 ± 30.7 and 156.3 ± 26.6 kDa, respectively. Similarly, a D_{37} value of 4.4 ± 0.6 Mrads was measured for the acid–base induced pyrophosphate synthesis resulting in a radiation sensitive size of 196.3 ± 31.9 kDa.

Functional size; Radiation inactivation; Pyrophosphatase; Chromatophore; *Rhodospirillum rubrum*

1. INTRODUCTION

The membrane-bound inorganic pyrophosphatase (PPase) is of importance as it is an independent alternative coupling factor to the ATPase system [1]. The hydrolysis of inorganic pyrophosphate is associated with various energy-requiring reactions, such as ATP synthesis [2], cytochrome redox changes [3], carotenoid band shift [4], succinate-linked pyridine nucleotide reduction [5] and transhydrogenation [6].

The PPase of *Rhodospirillum rubrum* has been isolated to a state of near homogeneity by Baltscheffsky et al. [7,8]. The purified PPase could be incorporated into phospholipid vesicles and acted as a PP_i-dependent electric generator [7]. The purified PPase contained 6–7 discrete subunits as shown by sodium dodecylsulfate-polyacrylamide gel electrophoresis with apparent molecular masses of 64, 52, 41, 31, (25), 20, and 15 kDa, respectively [8]. Other properties of this isolated PPase have been characterized previously [7]. However, the exact structure and mechanism of PPase still remain to be determined.

The technique of radiation inactivation has been used effectively in estimating the native molecular weight of many membrane-bound components such as enzymes, transporters, and receptors [9–11]. The functional size analysis may offer a means to determine the composition and stoichiometry of the functional subunits. It also sheds light on the elucidation of the mechanism of many enzyme complexes. In this communication we determined the functional size of PPase from *R. rubrum* by the radiation inactivation technique.

Abbreviations: BChl, bacteriochlorophyll; PPase, pyrophosphatase; PP_i, inorganic pyrophosphate

Correspondence address: R.L. Pan, Institute of Radiation Biology and Nuclear Science, College of Nuclear Science, National Tsing Hua University, Hsinchu 30043, Taiwan, Republic of China. Fax: (86) (35) 719744.

2. MATERIALS AND METHODS

2.1. *Preparation of* Rhodospirillum rubrum

The *R. rubrum* S1 strain was a kind gift from Dr Z. Gromet-Elhanan, Department of Biochemistry, Weizmann Institute of Science, Rehovot, Israel. The growth conditions were as described by Bose et al. [12]. The cells were harvested at log phase and washed, and chromatophores were prepared as described by Nyren et al. [7]. BChl concentrations were determined using the in vivo extinction coefficient given by Clayton [13], $\epsilon_{880} = 140\ mM^{-1} \cdot cm^{-1}$.

2.2. *Enzyme assay*

The PPase activities from chromatophores of *Rhodospirillum rubrum* were assayed in a 0.3 ml medium containing 0.1 M Tris-HCl (pH 7.5), 0.75 mM $MgCl_2$, 0.5 mM Na-PP_i. After 10 min the reaction was stopped by adding 10 mM NaF. The phosphate released was determined according to LeBel et al. [14]. The H^+-translocation across the membrane was measured as the rate of fluorescence quenching of Acridine orange at 30°C using a Hitachi F-4000 fluorescence spectrophotometer [11]. The excitation and emission wavelengths were 468 nm and 538 nm, respectively. The assay medium contained 0.2 M glycylglycine (pH 7.4), 5 mM $MgSO_4$, 5 μM Acridine orange and 3.6 μM BChl. The reaction was started by adding PP_i to a final concentration of 100 μM and stopped by adding 1 μM gramicidine. The acid–base transition PP_i synthesis was carried out according to a modified method of Jagendorf and Uribe [15]. Bacterial chromatophores were incubated for 1 min in 0.3 ml acid buffer containing 15 mM succinate, 0.4 M glycylglycine buffer (pH 4.7). 0.4 ml base buffer was added for another 1 min. The base buffer contained 0.4 mM glycylglycine (pH 8.6), 5 mM Mg-acetate, 4 mM Na-

phosphate, 10 μg oligomycin, and 5 μCi/ml carrier-free $^{32}P_i$. The reaction was terminated by the addition of 0.4 ml 15% (w/v) trichloroacetic acid and then centrifuged at 7826 × g for 5 min. Aliquots of supernatant (0.8 ml) were washed with 1.2 ml acetone and left standing for 5 min. The [^{32}P]PP_i was determined according to a method modified from Avron [16].

2.3. *Irradiation of samples*

Radiation inactivation analysis of PPase was carried out according to the method of Pan et al. [11]. Chromatophores (0.5 mg/ml BChl) were frozen in a medium containing 0.1 M Tris-HCl (pH 7.5) and 10% glycerol. The samples were irradiated with ^{60}Co (~1000 Ci) for various periods at −13°C to −20°C maintained by a cryothermostat. The dose rates were approximately 1.02 ± 0.13 Mrads/h determined by a method of Hart and Fricke [17] using Fe^{2+}/Fe^{3+} or Ce^{3+}/Ce^{4+} couples. The irradiated chromatophores were assayed immediately or restored at −70°C until used.

2.4. *Calculation of functional size*

Functional size was calculated according to the equation of Beauregard and Potier [18]:

$$\log m = 5.89 - \log D_{37,t} - 0.0028\, t$$

where m is the functional size in daltons, t is the temperature (°C) during irradiation, $D_{37,t}$ is the dose of radiation in Mrads required to reduce the activity to 37% of that found in unexposed control at temperature t (°C).

3. RESULTS AND DISCUSSION

When isolated chromatophores were exposed to high energy γ-ray irradiation, the pyrophosphate hydrolysis was reduced with increasing radiation dose (Fig. 1). The decay of enzymatic activity was as a simple exponential function of dosage, allowing straightforward application of target theory for determination of functional mass involved. From analysis of data using linear regression ($r = 0.96$), it was found that the D_{37} value of 5.2 ± 0.7 Mrads was required to decrease the activity to 37% of control value for PP_i hydrolysis reaction. The D_{37} value yields a functional size of 167.7 ± 30.7 kDa according to the equation of Beauregard and Potier [18].

In parallel experiments, we investigated the dose–response of PP_i-driven H^+-translocation across the chromatophore membrane as determined by fluorescence quenching of ΔpH probe, Acridine orange (Fig. 2A). The rate of H^+-translocation also decayed as a simple exponential function of dosage (Fig. 2B). A D_{37} value of 5.8 ± 0.8 Mrads was measured ($r = 0.95$), yielding a functional mass of 156.3 ± 26.6 kDa. This value is very similar to that of PP_i hydrolysis.

In further, proton-pyrophosphatase of chromatophores from *R. rubrum* could catalyze the synthesis of PP_i at the expense of chemiosmotic gradient generated under illumination. To make the system simple, we determined the functional size for PP_i synthesis by the method of acid–base transition [15], bypassing the involvement of photosynthetic electron transport chain. Similarly, we obtained a D_{37} value of 4.4 ± 0.6 Mrads and a functional mass of 196.3 ± 31.9 kDa ($r = 0.95$) (Fig. 3). The functional size for PP_i synthesis is slightly

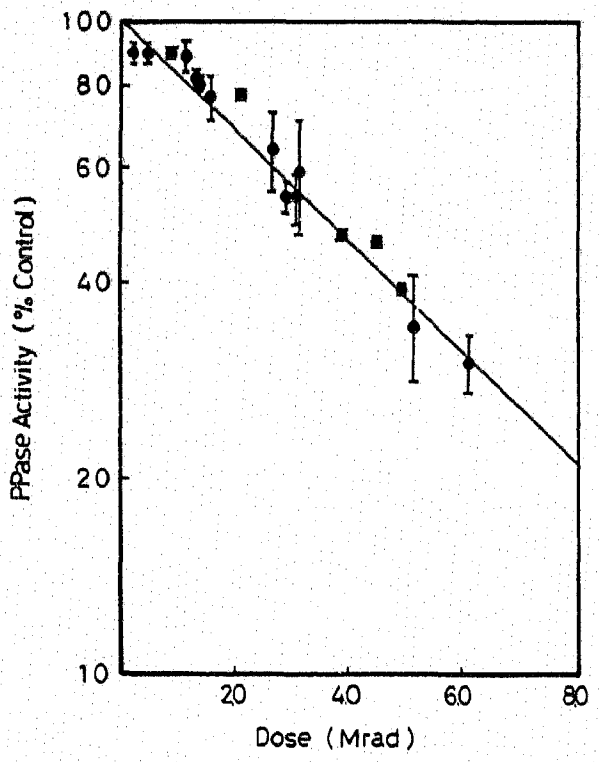

Fig. 1. Radiation inactivation of pyrophosphate hydrolysis by chromatophores of *Rhodospirillum rubrum*. Chromatophores of *Rhodospirillum rubrum* (0.5 mg/ml BChl) were irradiated and PP_i hydrolysis assayed as described in section 2. All data points are means of at least 3 assays with line fitted by regression analysis ($r = 0.96$). The intersect on line at 37% activity of control gives the D_{37} dose value. The control PPase activity was 2.75 μmol PP_i hydrolyzed/mg BChl/min.

larger than that for hydrolysis or its associated H^+-translocation.

Baltscheffsky et al. [8] demonstrated that PPase contained 6–7 discrete subunits on sodium dodecylsulfate polyacrylamide gel electrophoresis with apparent molecular weights of 64000, 52000, 41000, 31000, (25000), 20000 and 15000, respectively. It is believed that the 25-kDa polypeptide may not be a part of the enzyme since its band was usually very faint or even absent [8]. Thus the sum of molecular mass of the remaining subunits above is 223 kDa which is still larger than that determined by radiation inactivation in this report. This discrepancy might be interpreted as that some subunits play a structural role rather than being directly involved in enzymatic function. On the contrast, Klemme et al. obtained a molecular weight of 100000 for PPase by gel filtration [19]. It is likely that some subunits might be depleted during purification by Klemme et al. [19]. Recently, several PPases were isolated from higher plant vacuoles [20–23]. The molecular mass of one major polypeptide was in the range of 64–73 kDa upon sodium dodecylsulfate polyacrylamide gel electrophoresis. It was suggested

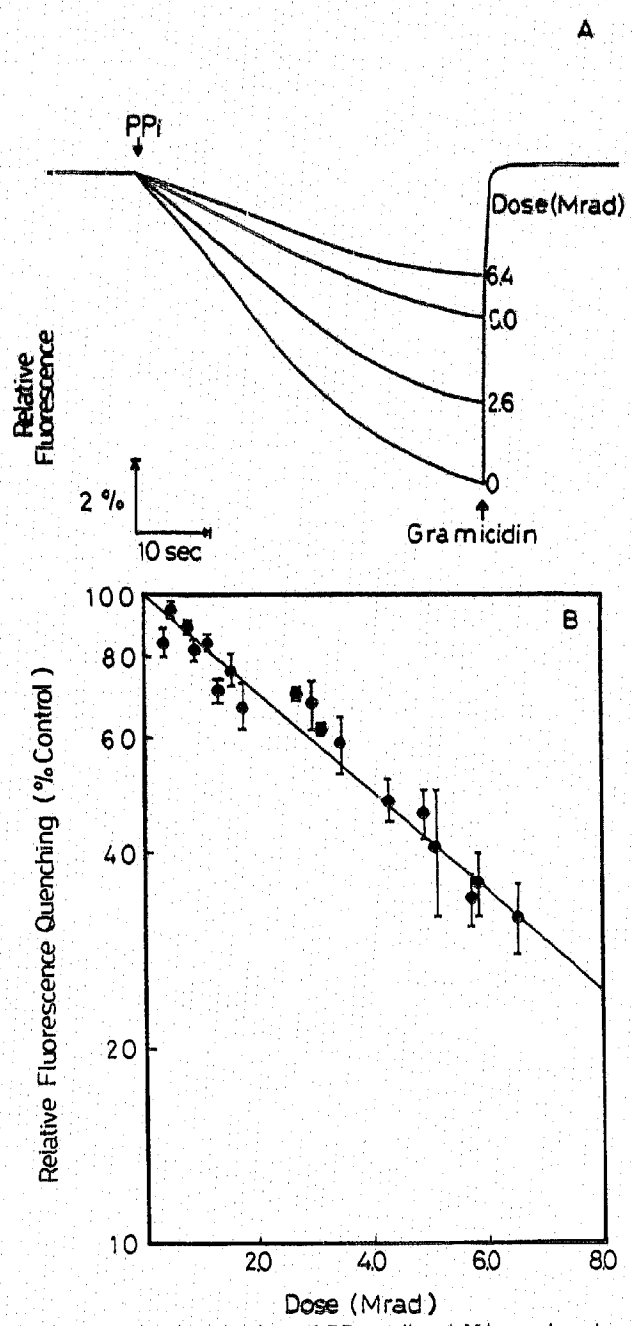

Fig. 2. Radiation inactivation of PP_i-mediated H^+-translocation of chromatophores from *Rhodospirillum rubrum*. The irradiation of chromatophores and the measurement of H^+-translocation were carried out as described in section 2. The initial rates of fluorescence quenching were determined from changes in fluorescence observed in first 30 s (A). One percent of fluorescence quenching represents 0.16 nmol proton influx determined by the direct titration of standard HCl. The data points in (B) are the means of at least 3 assays with the line fitted by regression analysis ($r = 0.95$). The line of intersect at 37% of control activity gives the D_{37} dose value.

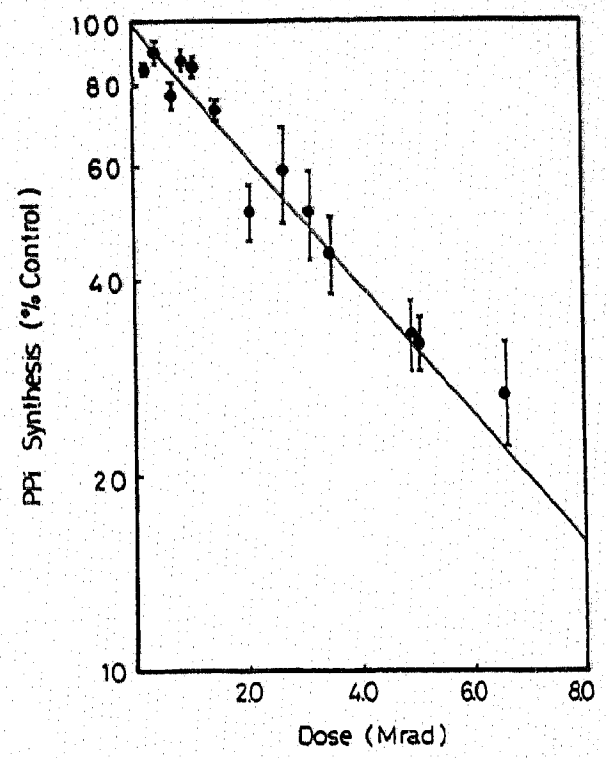

Fig. 3. Radiation inactivation of acid–base induced PP_i synthesis by chromatophores of *Rhodospirillum rubrum*. The irradiation of chromatophores and the reaction conditions of acid–base induced PP_i synthesis were as described in section 2. All data points are means of at least 3 assays with line fitted by regression analysis ($r = 0.95$). The intersect on the line at 37% activity of control gives the D_{37} dose value. The control activity of PP_i synthesis was 98 nmol PP_i formed/mg BChl/min.

that the structure of PPase on higher plant vacuoles may be an oligomer, probably a dimer [21]. We speculate the larger functional mass than that of individual subunits mentioned above indicates that PPase on chromatophores of *R. rubrum* may function as an oligomer in a manner similar to that on higher plant vacuolar membranes. The exact functional subunits and their composition are yet to be determined. Finally, the functional size of PPase is slightly larger for synthesis than for hydrolysis (cf. 196.7 ± 31.9 kDa versus 167.7 ± 30.7 kDa, respectively) implying that both mechanisms may not employ identical machinery. The elucidation of this possibility and the identification of components involved require further studies.

Acknowledgements: This work was supported by a grant from National Science Council, Republic of China to R.L.P. We thank Mr Fu Chuan Song of this institute and Mr San Te Sheu, Ping Yao Chen and Chia Lian Tseng of the Isotope Division, Nuclear Science and Technology Development Center, for the irradiation of samples and the technique assistance.

REFERENCES

[1] Baltscheffsky, M. and Nyren, P. (1984) New Comp. Biochem. 9, 187–206.
[2] Keister, D.L. and Minton, N.J. (1971) Arch. Biochem. Biophys. 147, 330–338.

[3] Baltscheffsky, M. (1967) Biochem. Biophys. Res. Commun. 28, 270–276.
[4] Baltscheffsky, M. (1969) Arch. Biochem. Biophys. 120, 646–652.
[5] Keister, D.L. and Yike, N.J. (1967) Arch. Biochem. Biophys. 121, 415–422.
[6] Keister, D.L. and Yike, N.J. (1967) Biochemistry 6, 3847–3857.
[7] Nyren, P., Hajnal, K. and Baltscheffsky, M. (1984) Biochim. Biophys. Acta 766, 630–635.
[8] Baltscheffsky, M. and Nyren, P. (1986) in: Methods in Enzymology, Vol. 126 (Fleischer, S. and Fleischer, B. eds) pp. 538–545, Academic Press, New York.
[9] Hutchinson, F. and Pollard, E. (1961) in: Mechanisms in Radiobiology (Errera, M. and Forssberg, A. eds) pp. 71–92, Academic Press, New York.
[10] Kempner, E.S. and Schlegel, W. (1979) Anal. Biochem. 92, 2–10.
[11] Pan, R.S., Chien, L.F., Wang, M.Y., Tsai, M.Y., Pan, R.L. and Hsu, B.D. (1987) Plant Physiol. 85, 158–163.
[12] Bose, S.K., Gest, H. and Ormerod, J.G. (1961) J. Biol. Chem. 236, 13–14.
[13] Clayton, R.C. (1963) in: Bacterial Photosynthesis (Gest, H., San Pietro, A. and Vernon, L.P. eds) pp. 495–500, Antioch Press, Yellow Springs, OH.
[14] LeBel, D., Poirier, G.G. and Beaudoin, A.R. (1978) Anal. Biochem. 85 86–89.
[15] Jagendorf, A.T. and Uribe, E. (1966) Biochemistry 55, 170–177.
[16] Avron, M. (1960) Biochim. Biophys. Acta 40, 257–272.
[17] Hart, E.J. and Fricke, H. (1967) in: Chemical Dosimetry, pp. 167–239, Academic Press, New York.
[18] Beauregard, G. and Potier, M. (1985) Anal. Biochem. 150, 117–120.
[19] Klemme, J.H. and Gest, H. (1971) Eur. J. Biochem. 22, 529–537.
[20] Maeshima, M. and Yoshida, S. (1989) J. Biol. Chem. 264, 20068–20073.
[21] Maeshima, M. (1990) Biochem. Biophys. Res. Commun. 168, 1157–1162.
[22] Britten, C.J., Turner, J.C. and Rea, P.A. (1989) FEBS Lett. 256, 200–206.
[23] Safrafian, V. and Poole, R.J. (1989) Plant Physiol. 91, 34–38.