AGING

Indian Perspective and Global Scenario

Edited by

Vinod Kumar

Professor of Medicine
All India Institute of Medical Sciences
New Delhi, India

Proceedings of the International Symposium on Gerontology and Seventh Conference of the Association of Gerontology (India)

1996

117

KINETIC DIFFERENCES IN LIVER INORGANIC PYROPHOSPHATASE DURING DEVELOPMENT OF CHICK

Don Kupar Syiem and Ramesh Sharma

INTRODUCTION

Development and aging are universal phenomena at two ends of an organism's lifespan and in between is the reproductive phase or adulthood. The time of onset, duration and rate of each phase is appropriately controlled. Though several important findings have been made in the elucidation of the problems of development, much remains to be explored for a proper understanding of the mechanism of development as well as aging, these being interrelated processes. Several studies have been conducted on organisms of different complexities, to answer specific questions related to development and/or aging.

All biological reactions in an organism are catalyzed by enzymes and these are therefore, essential for all functions of the body. They are the main molecular phenotypes that represent specific genotypes of the organism. The initiation, duration and termination of various phases in the life of an organism such as differentiation, development and maturity may depend on various characteristics of enzymes, such as levels, isoenzyme patterns and changes in their physico-chemical properties. Much data have accumulated and have been reviewed,[1,2,3] focussing on such changes. The levels of certain enzymes may decrease, others may increase and some remain unchanged. These changes may be tissue, species and/or class specific. A large body of evidence also suggests that changes in the activities and stabilities of numerous enzymes may primarily be due to post-translational modifications.[4] Many of these changes have been observed both in the housekeeping proteins[5,6] and in proteins involved in genetic information transfer.[7]

Enzymes are also known to be influenced by several effectors such as substrates, metabolites and ions in the cell. They constitute a regulatory mechanism which is necessary to coordinate a complex series of reactions in the body. The internal milieu within a cell may change during the course of development and aging; contiguous with this change, the activities of several enzymes may also undergo physical or functional alteration. In this paper we studied inorganic pyrophosphatase, an enzyme which is controlled by endogenous effectors, such as substrate and metal ions.[8] PPi is produced in a variety of cell metabolic processes such as, biosynthetic nucleoside triphosphate dependent reactions like deoxyribo- and ribonucleotide polymerization, activation of amino- and fatty- acids and coenzyme synthesis. It is apparently involved in diverse functions such as bone metabolism,[9] oxidative processes in mammalian mitochondria[10] and respiratory and photosynthetic processes in plants,[11] and bacteria.[12] The high affinity of PPi for divalent metal ions makes it a natural chelator of these ions in vivo,[13] thereby regulating those enzymes which are activated or inhibited by such metal ions. It is evident therefore that intracellular concentration of PPi can be a significant metabolic regulator for many reactions within the cell, and that the enzyme which splits PPi may play an important role in regulating metabolic processes.

The breakdown of PPi is carried out by specific inorganic pyrophosphatase (EC 3.6.1.1; PPiase) which is ubiquitous in nature. The enzyme has been extensively studied and reviewed in case of E coli and yeast,[14, 15] and it has also been purified from some plants and mammals. On the basis of subunit structure and cellular localization, two forms of PPiase have been shown to exist, membrane bound and cytosolic in mammalian system.[16] We chose to study the levels and kinetic properties of cytosolic inorganic pyrophosphatase at two different ages (immature and mature) of male chick Rhode Island Red breed.

MATERIALS AND METHODS

Materials: Male chicken of Rhode Island Red (RIR) breed, of 5 and 90 days old were used for the experiment. Tetra sodium pyrophosphate, malachite green, tris-hydroxymethylaminomethane, dithiothreitol, 2-mercaptoethanol and sephadex G-25 were from sigma. The other chemicals were of analytical grade.

Enzyme preparation: A 10% (w/v) homogenate of liver was prepared in ice cold 0.25 M sucrose using a glass tube and motor driven teflon pestle. The homogenate was centrifuged at 27,000 g for 60 min at 2°C. The resultant supernatant was used for assay of enzyme activity. Further the cytosol was dialyzed against 50 mM tris-HCL, pH 8.0, containing 10 mM 2-mercaptoethanol and 5 mM EDTA overnight. in the cold to remove endogenous metal ions. Subsequently the dialyzate was passed through a small sephadex G-25 column to remove EDTA and the eluants with maximum activity were pooled and used for kinetic studies.

Assay of PPiase: Using a slightly modified procedure of Weinhouse[17] malachite green assay of orthophosphate, the enzyme was measured. Appropriately diluted enzyme preparation was added to assay buffer (50 mM tris-HCl, pH 8.0, containing 1 mM DTT and 1 mM $MgCl_2$). The reaction was started by adding 0.1 mM substrate to the reaction

Int'l Symp Gerontol & VIIth Conf. AGI
Nov. 14-16, 1994. AIIMS ed. V. Kumar

Balaji Printers. New Delhi-55
1996

mixture and after 15 min of incubation at 25°C, tubes were transferred to an ice bath. Residual reaction was terminated by adding 2.4 M perchloric acid. Molybdate -malachite green color reagent was then added and color allowed to develop in the cold was monitored at 660 nm (λ) using a hitachi model U-2000 spectrophotometer. One unit of enzyme is defined as that amount which liberates 1 µmole of orthophosphate per minute, and that of specific activity as the unit of enzyme per mg protein. $K_2 HPO_4$ was used routinely as reference standard for each assays.

Protein estimation: Protein concentration of samples was determined by the method of Bradford[18] using bovine serum albumin as reference standard.

Kinetic characterization: Chicken liver cytosol of both the ages (5 and 90 days) were dialyzed overnight in the cold, passed through sephadex G-25 and eluate used for PPiase assay at varying [PPi] and [Mg2+] concentration. Per cent inhibition effect of Ca2+ and inactivation by guanidine hydrochloride (Gu HCl) was studied at various concentrations of these effectors. Effect of pre-incubation at different temperatures on PPiase was conducted at 4, 25 and 35°C.

RESULTS AND DISCUSSION

Soluble inorganic pyrophosphatase (PPiase) shows a higher level (20 %) in the liver of 5 day old chicken as compared to 90-day old, with specific activity of 1.6 ± 0.18 and 1.28 ± 0.073 respectively. Cytosol dialyzed against buffer containing EDTA however, showed lower, but a greater difference (60 %) in activity of PPiase between the two ages (Fig.1). The higher level of PPiase at day 5 may contribute to higher anabolic activity of the liver at this phase of chicken development. Several metabolic adjustments take place during early postnatal development of animals.[19] As PPiase is involved in biosynthetic processes[20] its greater level may provide better adjustments during early phases of development. Substrate saturation studies (Fig.2A) with PPi show sigmoidal patterns with apparent S 0.5 values of 15.8 µM and 17.7 µM respectively for 5 and 90 day old chicken liver PPiase. Hill plots of the data gave a hill coefficient (nH) values of 1.5 µM and 1.2 µM respectively at these two ages studied which may indicate a slight allosteric difference. It seems 5 day old chicken liver PPiase has greater affinity for the substrate than that of 90 day old enzyme. A similar change in specific activity of muscle glyceraldehyde-3 phosphate dehydrogenase was reported by Gafni.[21] PPiase activity of chicken liver for both ages show maximum activity at 25 mM $MgCl_2$+ concentration but with 5-day enzyme having a much higher specific activity (0.8 U/mg) than 90-day old (0.3 U/mg). Excess Mg2+ (50 mM), inhibits the PPiase activity of both ages, but the 60 per cent difference in activity was still observed. The actual substrate of inorganic PPiase being MgPPi complex, inhibition by Mg2+ as well as PPi (data not shown) at higher concentration conforms to homotropic as well as heterotropic regulation as reported for mouse cytosolic enzyme.[13] As shown in Fig 2B, the activity of 5-day PPiase enzyme at .01 M Mg2+ concentration was only 18 % compared to that observed at optimum Mg2+ concentration (25 mM), whereas the 90 day PPiase enzyme activity was 33 %. Changes in liver magnesium will thus affect 5 day old chicken liver PPiase more than that of 90 day. The 5 day PPiase enzyme also shows higher thermal stability compared to day 90 (Fig. 3). In addition, calcium inhibition studies revealed lower per cent inhibition of PPiase activity at day 5 compared to that of 90 day old chicken. A 50 % inhibition of activity was observed at 0.47 mM and 0.09 mM calcium, with 5 and 90 day old chicken, respectively (fig. 4A). Physiological role of Ca2+ in regulating endogenous activities of PPiase has not been

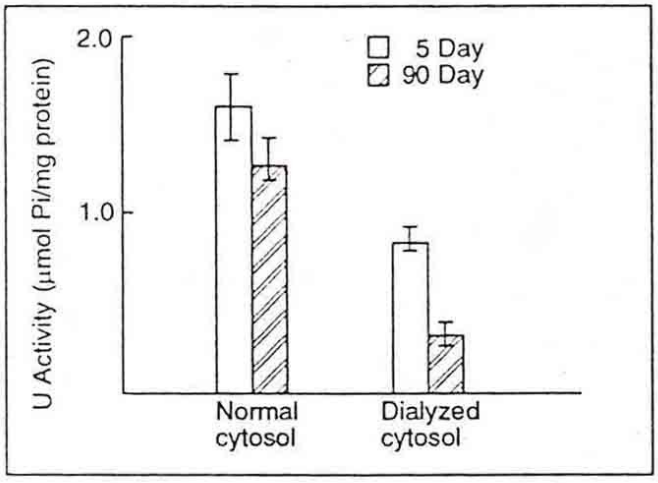

Fig. 1. Activity of Liver inorganic pyrophosphatase in normal and dialyzed cytosol of 5-(□) and 90- (▨) day old chicken. Fractionation and assay procedure are discussed in materials & methods. Values are means for 3-4 chickens of each age group. Bar represents standard deviation.

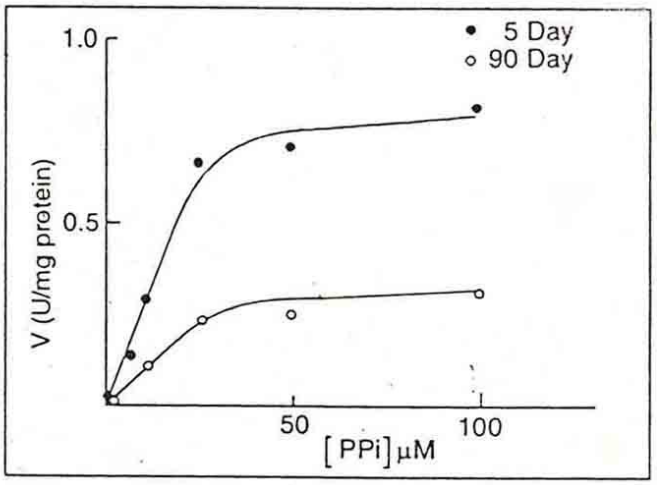

Fig. 2. (A) Effects of substrate (pyrophosphate) on the activities of liver Ppiase from 5-day (●) and 90-day (○) old chicken liver. The cytosols were dialyzed and used for activity measurements at various substrate concentration.

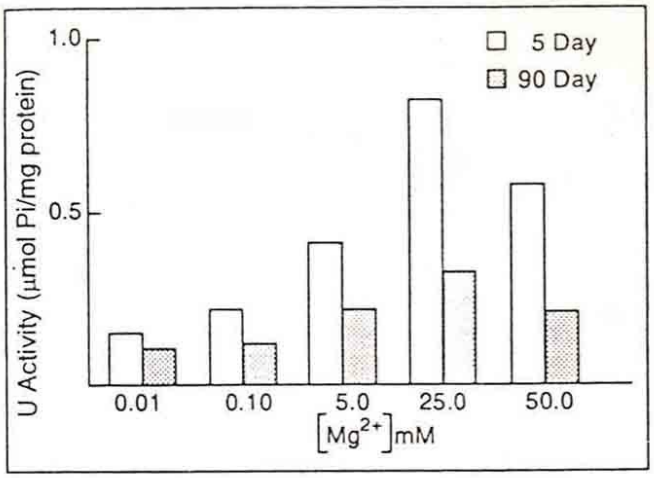

Fig. 2. (B) Effect of varying Mg2+ concentration on the activity of liver PPiase at optimum substrate concentration on 5-(□) and 90-(▨) day. Experimental procedures are the same as above.

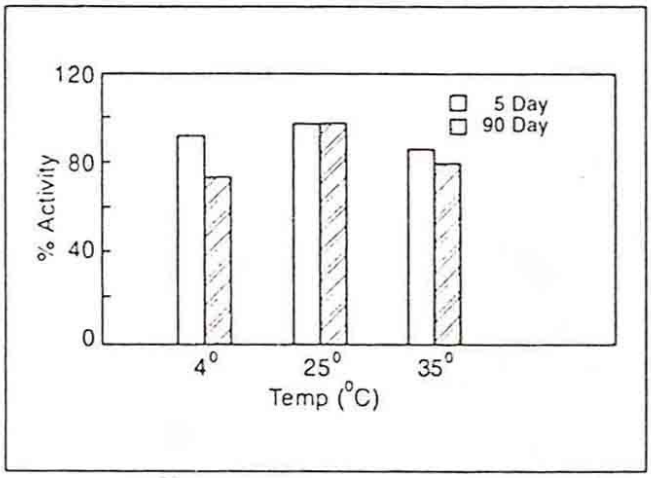

Fig. 3. Effect of temperature on the activity of PPiase from the liver of 5-(□) and 90-(▨) day old chicken. Enzyme preparation was preincubated at temperature shown in the figure and then assayed at optimum standard conditions.

elucidated but increase in matrix Ca2+ concentration has been implicated with increase in matrix PPi concentration with concommitant increase in mitochodrial volume which stimulates respiratory activity.[22] The greater protection of the soluble chicken liver PPiase against Ca2+ dependent inhibition at day 5 however, may ensure its better role of anabolic requirement needed in developing chicks. Similar trends of inactivation at 0.58 and 0.37 M guanidine hydrochloride (Fig. 4B) was achieved for day 5 and 90, indicating greater nonpolar interaction of amino acids in 5 day old chicken liver PPiase compared to 90 day old chicken enzyme.

Chelating agent like EDTA reduces residual activity of PPiase to a minimum and therefore, working with such a metal depleteing enzyme preparation makes possible to establish

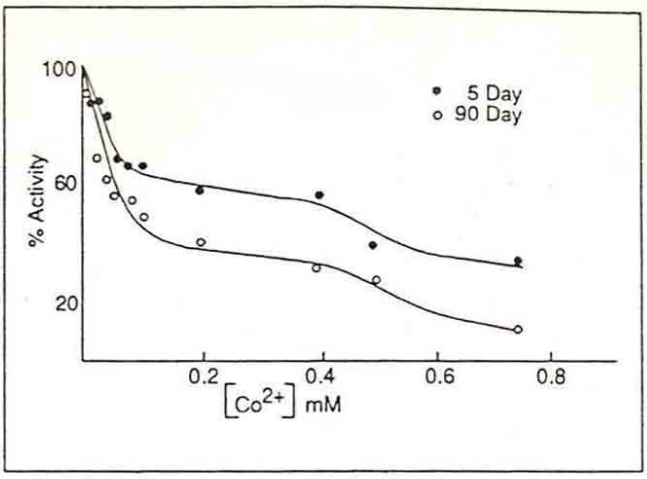

Fig. 4. (A) Inhibition of liver PPiase by calcium (Ca2+) in 5-(●) and 90-(○) day.

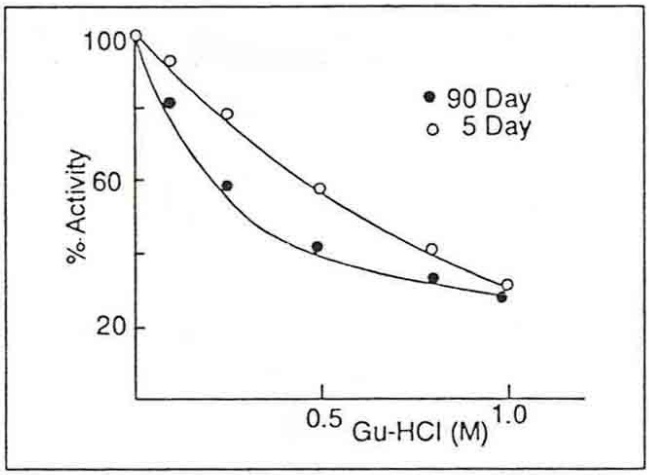

Fig. 4. (B) guanidine HCl (Gu HCl) inactivation in 5-(○) and 90-(●) day old chicken. Varying concentration of Ca2+ (A) and Gu HCL. (B) were used to assay the inhibition of PPiase at optimal conditions of assay.

the absolute dependence on added metal ions.[23] Our findings which show the enhanced activity difference between the two ages after dialyzing against EDTA containing buffer is a direct reflection of the role played by metal ions in regulating the activity of PPiase at these ages studied. From the above study we infer that the PPiase activity and stability with various modulators are greater in early stage of chicken development, giving more adaptive role for this constitutively expressed enzyme during development.

SUMMARY

Inorganic pyrophosphatase (PPiase) catalyses the hydrolysis of inorganic pyrophosphate (PPi) into two molecules of orthophosphate (Pi). It is involved in several biosynthetic pathways of proteins, nucleic acids and fatty acids synthesis. Keeping in view, this crucial role in the above processes, we chose to study the levels of this enzyme at two different

ages of male chicken. The result shows a small drop in the activity of PPiase in 90-day old compared to 5-day old chicken. Cytosol dialyzed against EDTA however, shows marked difference in the activity. Substrate saturation kinetics of dialyzed enzyme shows lower S in 5-day old compared to that of 90-day old chicken. 5-day old chicken liver enzyme shows lower inactivation by temperature, calcium and guanidine-HCI compared to 90-day old PPiase enzyme. These results suggest kinetic difference between the PPiase of the two age groups where early age PPiase being more active and more protected against various inhibitors, indicates better adaptive role for such a constitutively expressed enzyme at the early phase of chicken lifespan.

Acknowledgement

We would like to thank the Department of Science and Technology, New Delhi and the N.E.H.U. (North Eastern Hill University), Shillong for providing research facilities. D.K.S is also grateful to the Department of Biochemistry, N.E.H.U. for all its assistance.

REFERENCES

1. Kanungo MS. Biochemistry of Ageing. Academic Press, London 1980.
2. Sharma R. Enzymatic changes during aging. In: Timiras PS (ed). Physiolgical basis of aging and geriatrics. Macmillan Press, New York, pp 75-86, 1988.
3. Gafni A. Age related effects in enzyme metabolism and catalysis. In: Rothstein M (ed). Review of biological research in aging, vol 4, Wiley-Liss, New York, pp 315-336, 1990.
4. Rothstein M. Enzymes and altered proteins. In: Rothstein M (ed). Biochemical approaches to aging, Academic Press, New York, pp 213-255, 1982.
5. Toussaint C, Lemercier BB, Garlatti M, Hanoune J, Barouki R. Testis-specific transcription start site in the aspartate amino transferase housekeeping gene promoter. J. Biological Chemistry. 1994, 269: 3318-24.
6. Hoang T, Bergeron M. Adenosine deaminase in renal ontogeny and compensatory hypertrophy in the rat. Cell Tissue Kinet. 1983, 16: 59-64.
7. Kanungo MS. Phenotypic changes during aging. In: Kanungo MS (ed). Genes and Aging. Cambridge Press, New York, pp 21-54, 1994.
8. Want P, Strittmatter F. Characterization of a soluble inorganic pyrophosphatase from chicken liver. Int. J. Biochem. 1975, 6: 9-15.
9. Caswell AM, Russel RGG. Evidence that ectonucleoside triphosphate pyrophsphatase serves in the generation of extra cellular inorganic PPi in human bone and articular cartilage. Biochem. Biophys. Acta. 1988, 966: 310-317.
10. Volk SE, Baykov AA. Isolation and subunit composition of membrane inorganic pyrophosphatase from rat liver mitochondria. Biochem. Biophys. Acta. 1984, 791: 198-204.
11. Kowalezyk S, Maslowski P. Effect of inorganic pyrophosphate on respiration and oxidative phosphorylation in higher plants. Phytochemistry. 1981, 20: 2611-15.
12. Pal N, Nore BF, Baltsheffsky M. Studies on photosynthetic inorganic PPi formation in Rhodospirillum rubrum chromatophores. Biochem. Biophys. Acta. 1986, 851: 276-282.
13. Horn A, Bornig H, Thiele G. Allosteric properties of the Mg2+ -dependent inorganic pyrophosphatase in mouse liver cytoplasm. Eur. J. Biochem. 1967, 2: 243-249.
14. Josse J, Wong SCK. Inorganic pyrophosphatase of Escherichia coli. In: Boyer PD (ed) The Enzymes. 3rd edn., vol IV, Academic Press, New York, pp 499-527, 1971.
15. Gonzalez MA, Cooperman BS. Glutamic acid-149 is important for enzymatic activity of yeast inorganic pyrophosphatase. Biochemistry. 1986, 25: 7179-85.
16. Unguryte A, Smirnova IN, Baykov AA. Kinetic models for the action of cytosolic and mitochondrial inorganic pyrophosphatases of rat liver. Arch. Biochem. Biophys. 1989, 273: 292-300.
17. Shatton JB, Ward C, Williams A, Weinhouse S. A microcolori metric assay of inorganic pyrophosphatase. Anal. Biochem. 1983, 130: 114-119.
18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 1976, 72: 248-254.
19. Sharma R, Dey S, Verma R. Age-dependent development of malate-aspartate shuttle in the liver and kidney of mice. Biochem. Intl. 1992, 27: 1059-66.
20. Shatton JB, Williams A, Weinhouse S. Subcellular distribution of inorganic pyrophosphatase activity in various normal and neoplastic cell types. Cancer Res. 1983, 43: 3742-47.
21. Gafni A. Purification and comparative study of glyceraldehyde-3-phosphate dehydrogenase from the muscles of young and old rats. Biochemistry. 1981, 20: 6035-41.
22. Davidson AM, Halestrap AP. Inhibition of mitochondrial matrix inorganic pyrophosphatase by physiological [Ca2+] and its role in the hormonal regulation of mitochondrial matrix volume. Biochem. J. 1989, 258: 817-821.
23. Mildvan AS. Metals in enzyme catalysis. In : Boyer PD (ed). The Enzymes. Academic Press, New York, pp 446-536, 1970.