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In Vitro and in Vivo Age-Related Modification of Human Erythrocyte Phosphoribosyl Pyrophosphate Synthetase†

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ABSTRACT: Upon storage, human erythrocyte phosphoribosyl pyrophosphate synthetase (PRibPP synthetase, EC 2.7.6.1) from normal individuals was found to undergo a spontaneous dissociation into active enzyme components of much smaller molecular mass (60 000–90 000). These modified forms of enzyme exhibit kinetic properties different from the original large molecular weight enzyme (over 200 000). The small active components can be reversibly associated to form larger molecules in the presence of purine ribonucleotides as well as phosphoribosyl pyrophosphate (PRibPP). ATP was found to be most effective in associating PRibPP synthetase, while guanylate nucleotides seem to have no effect. The large molecular weight components, once separated from the milieu, were not able to undergo further dissociation. Fresh or stored human white cell or tissue homogenates were found to lack the low-molecular-weight enzyme under all our experimental conditions. A characteristic enzyme modification similar to that observed in stored erythrocyte was also noted in erythrocytes of increasing ages. The physiological significance of these findings to the regulatory function of PRibPP synthetase in purine metabolism in vivo is discussed.

Phosphoribosyl pyrophosphate synthetase (ATP:D-ribose-5-phosphate pyrophosphotransferase) catalyzes the synthesis of PRibPP from ATP and ribose 5-phosphate. This enzyme has been shown to be regulated by a complex mechanism in bacteria (Switzer, 1969, 1970, 1971), in rat liver (Roth et al., 1974a,b), and in man (Fox and Kelley, 1972). The enzyme is able to exist in many active forms of various molecular weights (Schubert and Switzer, 1975; Roth et al., 1974a,b; Fox and Kelley, 1971). A detailed study on the relationship of the PRibPP synthetase activity to its subunit association has been reported recently (Becker et al., 1977; Meyer and Becker, 1977). Reassociation of the enzyme molecules in vitro can be achieved by the addition of ATP. The current study is designed to clarify whether aggregation or dissociation of the native enzyme occurs in normal erythrocytes during the in vitro aging process and to explore the physiological significance of these changes as well as the regulatory nature of purine nucleotides and other metabolites on PRibPP synthetase.

Materials and Methods

All the nonradioactive chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. Radioactive substrates were purchased from New England Nuclear, Boston, Mass.

PRibPP Synthetase Enzyme Assay. Heparinized blood obtained from normal subjects was centrifuged at 1400*g* in an International refrigerated centrifuge for 10 min at 4 °C. The cells were washed several times with isotonic saline. After each washing, the buffy coat and the top layer of erythrocytes were carefully removed. The erythrocytes were hemolyzed by mixing with 2 volumes of distilled water, freezing in a dry ice–ethanol bath, and thawing three times. The mixture was then centrifuged at 25 000*g* at 4 °C in a Sorvall refrigerated centrifuge for 10 min, and the supernatant solution was used for the enzyme assay. The enzyme activities were measured by: (1) the amount of PRibPP produced (Yip and Balis, 1975a), (2) the conversion of [^{14}C]ATP to [^{14}C]AMP (Yip and Balis, 1975a) in the presence of ribose 5-phosphate, and (3) the reverse reaction of the conversion of [^{14}C]AMP to [^{14}C]ATP in the presence of phosphoribosyl pyrophosphate (Johnson et al., 1974). The assay mixture of (1) contained 70 mM Tris buffer (pH 7.4), 0.7 mM ethylenediaminetetraacetate, 1.7 mM mercaptoethanol, 7 mM magnesium ion, 33 mM potassium phosphate (pH 7.4), 0.17 mM ribose 5-phosphate, 0.17 mM ATP, 0.125 mM [^{14}C]adenine (sp act. 3.3 Ci/mol), and 5 μL of partially purified APRibTase (sp act. of 0.2 IU/mg, ca. 850-fold purified from human erythrocytes and completely void of PRibPP synthetase activity) in a total volume of 125 μL. Each sample was incubated in a 37 °C shaker bath for 10 to 60 min, depending on the amount of enzyme used. The reaction was terminated by immersion of the test tubes in an ethanol solid CO_2 bath. The procedure for the recovery of the radioactive nucleotide thus formed has been described (Yip and Balis, 1976). The assay mixture in 2 is essentially the same as that in 1, except [^{14}C]ATP (sp act. 0.8 Ci/mol) was used in place of [^{14}C]adenine and no purified APRibTase was added. Anion-exchange paper chromatography was used to separate the radioactive product from the reactants. After the

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termination of the reaction, the mixture was boiled for 2 min in a boiling water bath. Twenty-five microliters of the clarified reaction mixture was spotted on DEAE-cellulose paper (DE-81). After the additions of unlabeled markers, chromatography was carried out in an ammonium formate solution of pH 3.1, 0.6 M in concentration (Morrison, 1968) for 4 h. AMP and ADP spots were cut out and radioactivity was determined. Assay blanks had everything, including hemolysate and enzyme preparation, except ribose 5-phosphate. In the crude hemolysate assay, the reaction of adenylate kinase was taken into consideration; therefore, the activity of PRibPP synthetase is the sum of the radioactivity obtained from the AMP spot and half of that obtained from the ADP spot after each was corrected for its respective blank. The enzyme activity from the hemolysate obtained by either assay 1 or 2 was always in agreement. For the reverse reaction (3), [^{14}C]AMP (sp act. 0.67 Ci/mol) and PRibPP were used in place of [^{14}C]ATP and ribose 5-phosphate. Following the same procedure, the activity of enzyme reaction is the sum of the [^{14}C]ATP produced plus half of the radioactivity in the ADP spot, produced by the adenylate kinase reaction.

Storage of the Erythrocytes. The washed packed erythrocytes were generally stored at −20 °C without addition of protective agents. Under this condition, the loss of enzyme activity is negligible within the first 2 weeks. In one experiment, crude hemolysate (1:3 hemolysis) was stored at 4 °C with and without the addition of 0.3 mM ATP as a protective agent. The hemolysate without addition of ATP lost half of its enzyme activity in 48 h. No loss of enzyme activity was found with the hemolysate stored with ATP.

Molecular Weight Determination. The molecular weight of fresh and stored PRibPP synthetase was determined by gel filtration on a Sephadex G-200 column, using hemoglobin (65 000), rabbit muscle aldolase (158 000), and ovalbumin (45 000) as molecular weight standards. No attempt was made to differentiate enzyme molecules larger than 800 000 (in the void volume). The column (1.6 × 34 cm) was equilibrated with a buffer containing 0.18 M KCl, 1 mM 2-mercaptoethanol, 20 mM potassium phosphate buffer (pH 7.4), 1.5% glycerin, and 6 mM Mg^{2+}. About 0.6 mL of the crude hemolysate (1:3 hemolysis) was applied to the column, and ascending chromatography was run with the same buffer at 4 °C. The enzyme activity of the eluate was determined by using 25 μL of each of the 1 mL fractions. When a smaller Sephadex G-200 column (0.7 × 17.5 cm) was used, 100 μL of the sample was applied. Descending chromatography was used with 250 μL collected in each fraction. When the effect of purine ribonucleotides or other metabolites on the gel-filtration profile was studied, the concentration of the reagent in the buffer was 0.3 mM.

Purification of PRibPP Synthetase from Out-Dated Human Erythrocytes. The enzyme was partially purified by a modification of a method described previously (Yip and Balis, 1975a). The dehemoglobinized human erythrocyte lysate (from 1 L of outdated human blood) was chromatographed on a Sepharose 6B column (5.0 × 87 cm) twice, with and without 0.3 mM ATP in the elution buffer (0.18 M KCl, 0.02 M KPB (pH 7.4), 1 mM ETSH, 1.5% glycerin, 6 mM Mg^{2+}). After the second chromatography in the absence of ATP, two distinctly separated PRibPP synthetase peaks were obtained. The larger size had a molecular weight in the range of 1 000 000, and the smaller a molecular weight of 80 000. Fractions of large and small enzyme were pooled separately, concentrated by an amicon ultrafiltration method, and stored at −20 °C. The protein concentration of each concentrated pool was about 3 mg/mL.

Preparation of Human Leucocyte Lysate and Tissue Homogenate. Leucocytes were separated from 20 mL of fresh blood by the Ficoll-paque centrifugation method. The cells were washed by centrifugation three times with 3 mL of isotonic saline at 4 °C. Part of the washed leucocytes were stored as pellets at −20 °C until assay. The other part of the leucocyte was lysed immediately with 500 μL of distilled water with freezing and thawing. The clarified supernatant was used for the chromatography studies. Human tissue used in this study was obtained from the Tumor Procurement Center at the Memorial Sloan-Kettering Cancer Center. The 20% tissue homogenate was prepared as described previously (Yip and Balis, 1976).

Protein Determination. Protein concentration in each fraction was estimated by the absorption at 280 nm. Hemoglobin concentration was estimated by absorption at 577 nm. Total protein was determined by the method of Lowry et al. (1951).

Separation of Erythrocytes According to Age. Fresh normal human blood was collected, defibrinated, and washed according to the procedure described by Piomelli et al. (1967). Two milliliters of the packed erythrocytes was layered on top of the stractan gradient which had been prepared by the method of Corash et al. (1974). The tubes were centrifuged in the Spinco L-50 Beckman ultracentrifuge in the SW 27 rotor for 45 min at 51 800*g*. Five clearly separated bands of cells were observed after centrifugation. Each separate layer of cells was gently removed from the top of the gradient and was washed several times with isotonic saline before it was lysed and assayed.

Results

Changes of PRibPP Synthetase from Erythrocytes upon the in Vitro Aging Process. It is shown in Figure 1A that PRibPP synthetase from fresh normal human erythrocytes had a majority of its enzyme activity eluted in the void volume of a Sephadex G-200 column chromatogram. Only about one-fifth of the total recovered enzyme was observed to have a molecular weight near that of hemoglobin. Storage of the erythrocytes at −20 °C for 17 days caused a slight decrease in the enzyme activity. However, a drastic shift of the PRibPP synthetase molecular size was observed (Figure 1). At the end of 3 months only trace amounts of enzyme still possessed a molecular weight in the range of 800 000. Components of two distinct molecular weights, 60 000 and 90 000, were observed. When hemolysates were stored at 4 °C, a rapid change in both PRibPP synthetase activity and molecular size occurred. Figure 2 shows that fresh hemolysate (A) when stored at 4 °C had lost about 45% of its original activity in 48 h. The large-size molecule disappeared rapidly with a concomitant appearance of the smaller size enzyme molecule (Figure 2A, B). When ATP was added into the hemolysate during storage, but was not present in the chromatography buffer, preservation of the enzyme activity was observed (Figure 2C). However, ATP was not able to prevent dissociation of the enzyme molecule.

PRibPP Synthetase from Other Human Tissues. The enzymes from fresh human white cells, colonic mucosa, spleen, and liver cells have also been examined. Gel-filtration chromatography of homogenates of these cells showed the existence of only the large molecular weight components (Figure 3AB). Storage did not result in the dissociation of the enzyme molecule (Figure 3C,D). In the present studies, no attempt was made to differentiate the large-size molecules that were eluted at the void volume of the Sephadex G-200 column.

Effect of Storage on Partially Purified Large-Molecular-Weight PRibPP Synthetase. PRibPP synthetase was partially purified. The dehemoglobinized erythrocyte enzyme prepa-

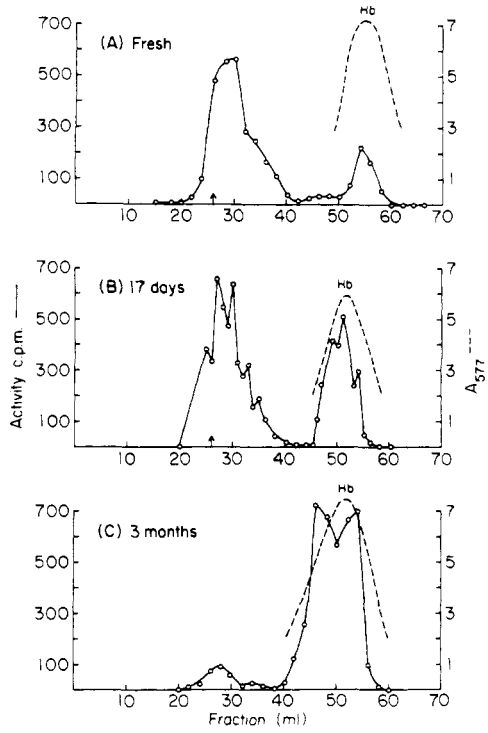

FIGURE 1: Changes in PRibPP synthetase from normal human erythrocytes upon storage: The molecular weight of fresh (A) and stored (B and C) PRibPP synthetase was determined by gel filtration on a Sephadex G-200 column. The column (1.6 × 34 cm) was equilibrated with a buffer containing 0.18 M KCl, 1 mM 2-mercaptoethanol, 20 mM potassium phosphate buffer (pH 7.4), 1.5% glycerin, and 6 mM Mg^{2+}. About 0.6 mL of the crude hemolysate of one normal individual (1:3 hemolysis of fresh erythrocytes and 1:2 hemolysis of stored erythrocytes) was applied to the column, and ascending chromatography was run with the same buffer at 4 °C. The enzyme activity (heavy line) of the eluate was determined as described in the text. Void volume of the column is seen by the position of the arrow. The position of hemoglobin elution is also shown (dotted line). Time of storage of the sample at −20 °C is indicated. The specific activity of the fresh erythrocyte PRibPP synthetase before chromatography, expressed as pmol of AMP produced (mg of protein)$^{-1}$ min^{-1}, was 724. No change of the specific activity was observed in 17 days at −20 °C. A loss of 40% activity was noted after 3 months of storage.

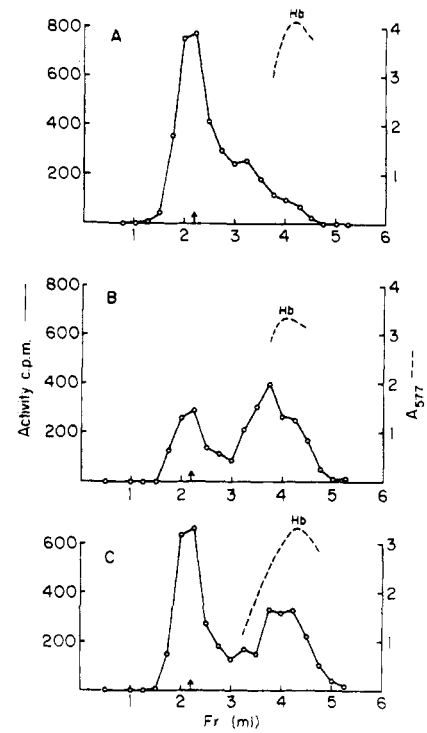

FIGURE 2: Effect of ATP on PRibPP synthetase upon storage. Crude hemolysate (1:3 hemolysis) was chromatographed (A) immediately after hemolysis, (B) 48 h after storage at 4 °C, (C) 48 h after storage at 4 °C in the presence of 0.3 mM ATP. Sephadex G-200 (0.7 × 17.5 cm) descending column chromatography was used. The sample applied was 100 μL. The column was equilibrated and eluted with the same buffer as described in Figure 1. Each fraction contained 250 μL, and 25 μL was used in the activity assay as described in the text. Before chromatography the specific activity of the sample in A [as expressed in pmol of AMP produced (*mg of protein*)$^{-1}$ *min*$^{-1}$] was *786, in B 464, and in C 806.*

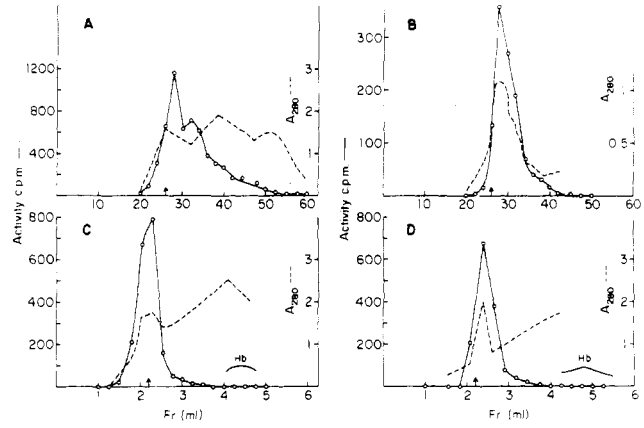

FIGURE 3: Gel-filtration chromatography behavior of fresh human leucocyte lysate (A), fresh human colonic mucosa homogenate (B), stored leucocyte lysate (C), and stored human spleen homogenate (D). The specific activities of the samples before chromatography in pmol of AMP produced (μg of protein)$^{-1}$ min^{-1} were: (A) 4.49, (B) 3.22, (C) 2.23, and (D) 1.91. The amounts of proteins of A and B applied to Sephadex G-200 column (1.6 × 34 cm) were 24 and 6.6 mg. Leucocytes and prepared tissue homogenates were stored at −20 °C for over 3 months before they were rechromatographed on a Sephadex G-200 column (0.7 × 17.5 cm). The amounts of protein applied were 4 and 2.2 mg each in 100 μL. The enzyme activity (solid line) and the protein profile of the eluates (dotted line) are shown in the graph.

ration was repeatedly chromatographed on a Sepharose 6B column with and without ATP. After the second chromatography on the Sepharose 6B column in the absence of ATP, two distinctly separate PRibPP synthetases were obtained. The larger size enzyme had a molecular weight in the range of 800 000. It was about 300-fold purified and was concentrated and stored at −20 °C at a protein concentration of 3 mg/mL for over 3 months before rechromatography on Sephadex G-200. No small-size enzyme was observed to appear after storage (Figure 4).

Gel-Filtration Profile of PRibPP Synthetase in the Presence of Purine Ribonucleotides and Related Metabolites. PRibPP synthetase has been reported to be an extremely labile enzyme. ATP was required to protect the enzyme from denaturation (Fox and Kelley, 1971). In our studies, chromatography of PRibPP synthetase on Sephadex G-200 without the presence of purine nucleotides gave 20–30% recovery of the enzyme activity. Recovery was increased to 70–90% with ATP, and to 47–60% with ADP. Little or no increase was observed with AMP or ITP (20–40%). A slight decrease of recovery (16–20%) was observed with 2,3-DPG, IMP, GMP, and GTP. PRibPP increased the recovery to 37%. Ribose 5-phosphate did not change the gel-filtration profile of the enzyme. The presence of ADP, ATP, or PRibPP caused the small components to reassociate into the larger molecules (Figure 5 and Table I). ITP was able to aggregate partially the small component. However, guanine nucleotides, 2,3-DPG and ribose 5-phosphate had no effect on enzyme size. Effects of nucleotides on reassociation were more prominently seen with stored

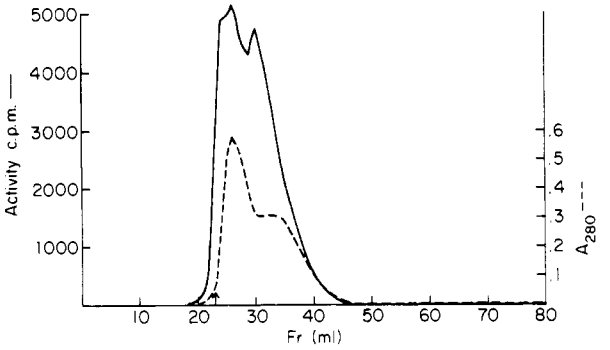

FIGURE 4: Gel-filtration chromatography profile of stored, partially purified PRibPP synthetase from human erythrocytes. PRibPP synthetase from out-dated human erythrocyte was purified according to the procedure described in the text. The high-molecular-weight enzyme fractions were pooled, concentrated, and stored at −20 °C in a buffer containing 0.18 M KCl, 1 mM 2-mercaptoethanol, 20 mM potassium phosphate buffer (pH 7.4), 1.5% glycerin, and 6 mM Mg^{2+}, at a protein concentration of 3 mg/mL. Specific activities of the enzyme before and after storage, expressed in pmol of AMP produced (μg of protein)$^{-1}$ min^{-1}, were 154.5 and 74.7, respectively. The enzyme activity (solid line) and protein profile (dotted line) of the partially purified PRibPP synthetase after 3 months of storage are shown in the graph.

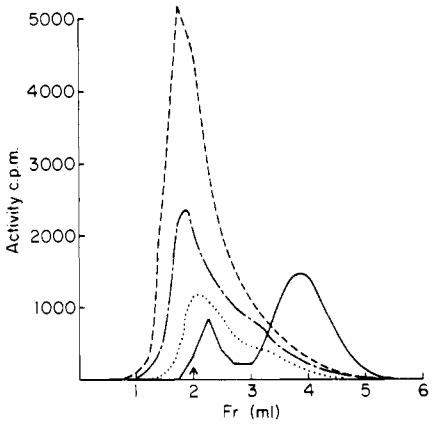

FIGURE 5: Sephadex G-200 chromatography of hemolysate PRibPP synthetase in the presence and absence of adenine ribonucleotide: Crude hemolysate (1:2 hemolysis) from stored human erythrocytes was chromatographed on a Sephadex G-200 column (0.7 × 17.5 cm) in the presence of 0.3 mM ATP (- - -), ADP (— —), or AMP (···) in the elution buffer. The control (solid line) contained no added nucleotides. The procedure of chromatography and enzyme activity assay were the same as described in Figure 2, except that the concentration of ATP used in the assay mixture was increased to 1.7 mM to counteract the inhibitory effect of the added nucleotides. Controls were assayed the same way. Enzyme activity recovery from the gel filtration was 24% with the control, 90% in the presence of ATP, 47% in the presence of ADP, and 20% in the presence of AMP.

erythrocytes. Since relative amounts of each form varied from batch to batch, a control was run with each experiment.

The effect of inorganic phosphate on the gel-filtration profile of PRibPP synthetase has been examined. The ratio of the large to small molecular weight components of the enzyme seemed to be independent of the amount of phosphate ion present in the elution buffer, i.e., from 5 to 200 mM. However, complete omission of the phosphate ion during the filtration processes led to extensive denaturation of the protein. Under such conditions, accurate measurement of the enzyme activity was not possible.

Inhibition of Large- and Small-Molecular-Weight PRibPP Synthetase by Purine Ribonucleotides and Related Metabolites. Among the four most effective promotors of PRibPP synthetase aggregation, i.e., ATP, ADP, AMP, and PRibPP

TABLE I: Sephadex G-200 Chromatography of Hemolysate PRibPP Synthetase in the Presence and Absence of Purine Ribonucleotides and Related Metabolites.[a]

treatment	enz distribut (% of total recov) large mol wt	small mol wt
control	55.6	44.4
+Rib-5-P	53.3	46.7
control	48	52
+2,3-DPG	53.5	46.5
control	36	64
+PRibPP	100	
control	32.2	67.8
+GTP	29.4	70.6
+GMP	31.3	68.7
control	30	70
+IMP	28	72
control	18	82
+ITP	79.3	20.7
control	16.7	83.3
+ATP	100	
+ADP	100	
+AMP	100	

[a] Normal stored human erythrocyte hemolysates (1:2 hemolysis) were used. The concentrations of purine nucleotides or other metabolites were the same, 0.3 mM, for all. The experimental details are described in Figure 5.

TABLE II: Inhibition of PRibPP Synthetase by Purine Nucleotides and Other Metabolites.[a]

enz prep	compd added ADP	AMP	IMP	2,3-DPG	GTP
lg mol wt eryth enz	114	113	111	109	107
sm mol wt eryth enz	78	88	90	94	96
spleen homogenate[b]	96	94			
white-cell lysate[b]	105	112			

[a] Values are given as: (activity in the presence of inhibitor/activity in the absence of inhibitor) × 100. Hemolysate (1:2) from stored erythrocytes (2 weeks at −20 °C) was chromatographed on a Sephadex G-200 column (0.7 × 17.5 cm). The two activity peaks were pooled separately (see Figure 1B). The enzyme from human spleen and white-cell homogenates after Sephadex chromatography (see Figure 3C,D) were also used in this experiment. The concentration of the nucleotides and metabolite added was 0.3 mM. Assays were performed as described in Figure 5. [b] After chromatography.

(Table I), ATP and PRibPP were either the substrate or the product of the reaction, which eliminates the possibility that complete inactivation of the small-molecular-weight enzyme was the result of the observed changes. Experiments were designed to see if the inhibitors, at the concentrations used for aggregation study, would differentially inhibit the various molecular weight forms. PRibPP synthetase from stored human erythrocytes, white cells, and spleen were chromatographed on Sephadex G-200. Active fractions of the large and small components were pooled separately and assayed in the presence and absence of the purine nucleotides and 2,3-DPG. Purine nucleotides and 2,3-DPG slightly activated the large enzyme but inhibited the small enzyme (Table II). This inhibition was most prominent with ADP. In general, at 6×10^{-5} M, under our experimental conditions, the changes were small but had a differential effect on large- and small-molecular-weight PRibPP synthetase.

TABLE III: Comparison of the Forward and Reverse Reactions of Large and Small Molecular Weight PRibPP Synthetase.[a]

enz sources	activity: pmol of AMP formed/h	activity: pmol of ATP formed/h	ratio, forward/reverse
crude hemolys before chromatogr	2160	1332	1.6
large mol wt eryth enz	812	213	3.8
small mol wt eryth enz	325	14	23.0
spleen homogen after chromatogr	2227	263	8.5
white-cell lys after chromatogr	1634	248	6.6

[a] The enzymes used in this experiment were the same as described in Table II. The forward and reverse reactions were assayed as described in the text. In enzyme activity assays, the crude hemolysate (1:2 hemolysis) was diluted 50× before use in the reaction. No dilutions were made with enzyme pools from Sephadex column chromatography.

TABLE IV: Age-Induced Modification of PRibPP Synthetase from Human Erythrocytes.[a]

samples[b]	enzyme act. (pmol of product (mg of Hb)$^{-1}$ min^{-1}): forward	enzyme act. (pmol of product (mg of Hb)$^{-1}$ min^{-1}): reverse	ratio forward/reverse
unfractionated	1290	830	1.56
fraction 1	1050	1340	0.78
fraction 2	790	810	0.97
fraction 3	955	300	3.18
fraction 4	712	125	5.86
fraction 5	627	210	3.00

[a] Fresh human erythrocytes were separated into five fractions of cells at different ages (see Materials and Methods). These cells were lysed in a minimum amount of 0.05 M Tris-HCl (pH 7.4) buffer with freezing and thawing at 4 °C. Hemoglobin concentrations and the catalytic activity for forward and reverse PRibPP synthetase reactions in each fraction were determined. [b] Samples increase in age from fraction 1 to 5.

Characteristic Difference between the PRibPP Synthetase of Large and Small Molecular Weight. The large enzyme from human erythrocyte, spleen, and white cells was more active in catalyzing the cleavage reaction than the small erythrocyte enzyme (Table III). The ratio of forward to reverse reaction of the latter was six times that of the large at comparable purity.

Modification of PRibPP Synthetase by the in Vivo Aging of Erythrocytes. Erythrocytes of different ages, separated by centrifugation in a discontinuous stractan gradient, have differences in their PRibPP synthetase activities (Table IV). A gradual loss of catalytic activity was observed with cells of increasing ages. However, since the ratio of the activity of the forward and reverse reactions was four to eight times greater in older cells (Fr 4 and 5) than in younger (Fr 1 and 2), an age-induced modification is evident. The mean of the fractions had the same ratio as unfractioned cells within experimental uncertainty.

Discussion

Native PRibPP synthetase has been found in multiple states of aggregation (Schubert and Switzer, 1975; Roth et al., 1974a,b; Fox and Kelley, 1971). The enzyme purified from outdated human erythrocytes in the presence of excess ATP, Mg^{2+}, and phosphate associates into two forms estimated to have molecular weights of 1 200 000 and 720 000. Upon removal of the substrate and effectors, it dissociates into components of 60 000 molecular weight (Fox and Kelley, 1971). Recently, Becker et al. (1977) have reported a detailed study of PRibPP synthetase subunit association and dissociation. However, they, as did earlier workers, purified the enzyme from out-dated erythrocytes in phosphate buffer with high concentrations of ATP and magnesium. The gel-filtration studies reported here show that native PRibPP synthetase from fresh human erythrocytes exists predominantly as the large-molecular-weight form regardless of the presence or absence of substrates and effectors. Upon storage it undergoes spontaneous dissociation into smaller active components. This process is slow at −20 °C, and there is concomitant loss in activity. ATP protects the enzyme from the loss of activity at 4 °C, but it is not able to prevent dissociation. Many metabolites, e.g., ATP, ADP, and 2,3-DPG, were found to vary in amount upon storage. Gel filtration effectively removes small molecules. Therefore, the differences between fresh and stored PRibPP synthetase after gel filtration in the absence of effectors cannot be the same subunit association and dissociation phenomenon that had been reported previously.

Unlike the erythrocyte enzyme, PRibPP synthetase from human leucocytes and other tissues does not undergo spontaneous dissociation. Roth et al. (1974a,b) have reported that native PRibPP synthetase from rat liver exists as an aggregated large molecule. Attempts at disaggregation by removal of substrates or phosphate were not successful. Only under drastic conditions, i.e., high concentrations of sodium chloride, did dissociation occur. These observations suggest an intrinsic difference between the erythrocyte enzyme and that in nucleated cells.

In most tissues, enzymes have relatively constant half-lives, which are the sum of the relative rates of synthesis and degradations. However, erythrocytes cannot synthesize protein and are incapable of renewing their enzymes. The original complement must function throughout the life of the cells, about 120 days. Consequently, a progressive modification of protein structure of many erythrocytes enzymes occurs with age (Yip et al., 1974). We have observed this with PRibPP synthetase. A gradual loss of the catalytic activity is seen with increasing age (Table IV). However, since the ratio of the forward to reverse reaction also changes with aging, a concomitant age-induced characteristic alteration of the protein molecule is evident. We have observed similar changes with storage in vitro.

One of the many theories postulated to explain age-related deterioration leading to senescence and death is that the accumulation of faulty protein molecules causes loss of functions, imbalance of metabolites, and consequently cell death (Wulf and Cutler, 1975; Gershon et al., 1974). We have previously reported that older erythrocytes have higher levels of PRibPP (Yip et al., 1974). Our current results on the PRibPP synthetase in aging erythrocytes suggest a possible mechanism behind that phenomenon. In view of the many regulatory roles of PRibPP, it is logical to consider the changes in the synthetase as a specific case of aging modification that is, at least in part, a result of changes in a functional protein. Of particular importance in this regard is the large change seen in the relative rates of the forward and reverse reaction.

PRibPP synthetase has been shown to be regulated in a complex manner depending on Rib-5-P availability, cellular energy levels, and end-product inhibition of the multiple pathways utilizing PRibPP (Switzer, 1969, 1970, 1971; Roth et al., 1974a,b; Fox and Kelley, 1972). The morphologically

different components of the enzyme differ not only in size but also in catalytic characteristics (Tables II and III). Substrates and products can regulate the size of the molecule (Table I). Therefore, the catalytic properties of the enzyme as it exists in vivo will most likely not be the same as those of the partially purified enzyme assayed in vitro. This may explain the observed catalytic difference between crude hemolysates and the enzyme partially purified by gel filtration.

In fresh erythrocytes PRibPP synthetase exists as a large-molecular-weight component which when separated from its native milieu no longer undergoes spontaneous dissociation. The specific enzyme protein concentration of the partially purified enzyme in storage was 0.46–0.22 unit/mL (1 unit = amount of enzyme able to produce 1 μmol of nucleotide/min), and that of the enzyme in intact erythrocytes is 0.3 unit/mL. Therefore, concentration cannot be a factor in the difference between crude and partially purified enzyme on storage.

Many purine metabolites are able to effect the reassociation of the low-molecular-weight enzyme (Table I). Among them, ATP and PRibPP are substrates (products) of the reactions. The fact that guanylates inhibit the enzyme but fail to induce aggregation suggests that PRibPP synthetase has multiple effector sites, as has been shown by others (Roth et al., 1974a,b). It may be relevant that AMP and GMP appear to produce different conformational alterations in pigeon liver amidophosphoribosyltransferase (Itoh et al., 1976). A simple explanation would be that GMP competes with AMP at the active site of the synthetase but not at the aggregation effector site.

Holmes et al. have shown that the intracellular concentration of PRibPP has a regulatory role in the biosynthesis of purine nucleotides in man (Holmes et al., 1973). It is an effector for APRTase (Yip and Balis, 1975a,b) and PRibPP amidotransferase. Binding of this compound alters the molecular weight of these enzymes. It acts as an aggregator as well as deaggregator of PRibPP amidotransferase (Itoh et al., 1976; Holmes et al., 1973). Our finding of its role as a promoter of PRibPP synthetase aggregation further emphasizes the significance of this metabolite as a regulator of purine metabolism. ATP is a substrate of PRibPP synthetase; it also induces aggregation of the small-molecular-weight erythrocyte enzyme. Therefore, the possibility that the dissociated product is actually an inactive enzyme molecule, yet appears to be active due to the presence of ATP in the assay mixture, cannot be completely ruled out.

Although the total activity in the forward direction (toward PRibPP synthesis) decreases with the age of the erythrocyte, the ratio of forward to reverse increases. This might explain the elevated PRibPP level in old red blood cells. In fact, the relative PRibPP concentration (Yip et al., 1974) parallels the ratio of forward to reverse and not the absolute enzyme level. Thus, the erythrocyte which is unable to make new enzyme can nonetheless control metabolism by programmed changes in size and, concurrently, activity of existing enzyme. One might, in view of somewhat similar changes seen in developing intestinal mucosal cells (Balis et al., 1971; Trotta and Balis, 1977), hypothesize that, in a more general sense, development and aging could be regulated by a certain specific physical modification in functional molecules that may or may not require synthesis of new mRNA and lead to altered metabolism.

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