Hypoxanthine–Guanine Phosphoribosyltransferase in Human Erythroid Cells: Posttranslational Modification†

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ABSTRACT: Hypoxanthine–guanine phosphoribosyltransferase (EC 2.4.2.8) (HGPRT) of human red blood cells has been separated into three major isoenzymes, the relative quantities of which change as the cell ages. The predominant isoenzyme in the youngest circulating red blood cells, reticulocytes, has the same isoelectric point as the single enzyme of lymphoblasts. This lymphoblast-like enzyme is diminished in older red cells, and the major fraction of HGPRT activity is recovered in the two more acidic isoenzymes. The HGPRT enzymes of human lymphoblasts and red cells have been purified to apparent homogeneity, as evidenced by the criterion of subunit molecular weight in $NaDodSO_4$ gels. The lymphoblast enzyme dissociates to a single subunit (α) upon isoelectric focusing in 8 M urea and is presumed to be a homo dimer ($\alpha\alpha$). The red cell isoenzymes dissociate to two subunits, one with the same isoelectric point as that in lymphoblasts (α) and one more negatively charged (α'). We infer that the three major red cell isoenzymes, I–III, correspond to enzyme species with none ($\alpha\alpha$), one ($\alpha\alpha'$), or both ($\alpha'\alpha'$) subunits modified. Tryptic peptide maps of these iodo[2-^{14}C]acetamide-labeled enzyme subunits indicate that the one red cell subunit (α) is identical with that in lymphoblasts and that the second subunit (α') differs from these in only one of the five cysteine-containing tryptic peptides. These results indicate that the HGPRT subunit is subject to at least one covalent and site-specific modification in human erythroid cells.

The single human structural gene for hypoxanthine–guanine phosphoribosyltransferase (EC 2.4.2.8) (HGPRT)[1] is located in the X chromosome (Nyhan et al., 1967; Rosenbloom et al., 1967a; Migeon et al., 1968; Nabholz et al., 1969). The HGPRT in all cell types may not be identical, however. Various methods yield multiple electrophoretic or isoelectric species of red cell HGPRT (Arnold & Kelley, 1971; Davies & Dean, 1971; Gulumian & Wakid, 1975; Bakay & Nyhan, 1975; Turner et al., 1975), while a single enzyme is found in white blood cells, fibroblasts, and lymphoblasts (Shin et al., 1971; Diggelen & Shin, 1974; Vasquez & Bieber, 1978; Zannis et al., 1980). Recent reports of comparisons of the enzymes purified from such cells have interpreted the changes leading to the formation of the red cell HGPRT isoenzymes as resulting from posttranslational modifications or enzyme aging (Ghangas & Milman, 1977; Zannis et al., 1980).

We have reinvestigated the basis for the occurrence of HGPRT isoenzymes in human erythrocytes. In the study reported here, red cell HGPRT has been compared with the enzyme in lymphoblasts. One of the red cell isoenzymes has the same isoelectric point as the single enzyme of lymphoblasts. In view of the possibility that the other red cell isoenzymes result from modification of the lymphoblast-like isoenzyme, the relative quantities of each isoenzyme have been estimated in red cells of differing ages. We describe methods for purification of the HGPRT enzymes from lymphoblasts and from red cells. Comparisons of the enzymes purified from the two cell types include specific activities of the active enzymes and the molecular weights and isoelectric points of the denatured subunits. Tryptic peptides of the iodo[2-^{14}C]acetamide-labeled HGPRT subunits are also compared.

Materials and Methods

(*1*) *Buffers.* The buffers used are identified by the following letters in the text: A, 10 mM phosphate (K), pH 6.8, with 0.25 M sucrose (Schwarz/Mann ultrapure) and 10 mM dithiothreitol (DTT) (Calbiochem); B, buffer A with 1 mg/mL bovine serum albumin (Sigma, crystalline); C, 10 mM phosphate (K), pH 6.8, 0.25 M NaCl, 0.1% Triton X-100, and 10 mM DTT; D, Dulbecco's phosphate-buffered saline without $MgCl_2$ or $CaCl_2$ (Dulbecco & Vogt, 1954); E, 1 mM phosphate (K), pH 7.9, 0.1 mM EDTA, and 0.1 mM DTT; F, 10 mM phosphate (K), pH 7.9, 0.1 mM EDTA, and 0.1 mM DTT; G, 10 mM phosphate (K), pH 6.5, 0.1 mM EDTA, and 0.1 mM DTT; H, 10 mM phosphate (K), pH 6.5, 1 mM EDTA, and 1 mM DTT; I, 10 mM phosphate (K), pH 6.8, 1 mM EDTA, 1 mM DTT, and 0.25 M NaCl; J, 10 mM phosphate (K), pH 6.8, 1 mM EDTA, and 8 M urea.

(*2*) *Growth of Lymphoblasts and Preparation of Lymphoblast and Red Blood Cell Lysates.* Lymphoblasts (PGLC 33H) (Glade et al., 1968) were grown in RPMI 1640 medium with 10–15% fetal calf serum. Human red cells were obtained from whole blood collected in the presence of heparin, EDTA, or CPD. Red cells of a Lesch–Nyhan patient lacking HGPRT activity were from CT, who is related to the family described by Nyhan et al. (1970). Red cells were obtained by removing the buffy coat at the red cell–supernatant interface. All cells were collected by centrifugation at 500*g* for 10 min at 4 °C. Lymphoblasts were suspended in at least 100 volumes of buffer D and resedimented 3 times. Red cells were rinsed 4 times with at least 5 cell volumes of buffer D. Cells were suspended in buffer A at approximately 10^7/mL for lymphoblasts and 10^8–10^9/mL for red blood cells. Cells were lysed by four cycles of freezing and thawing and stored at −20 or −80 °C.

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[1] Abbreviations: HGPRT, IMP:pyrophosphate phosphoribosyltransferase (EC 2.4.2.8); P-Rib-PP, 5-phosphorylribose 1-pyrophosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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(*3*) *Assays of HGPRT Activity.* Assays of HGPRT activity were carried out at pH 10 in 0.1 M glycine (Na) as previously described (Johnson & Littlefield, 1979). Product formation in this assay is proportional to the concentration of lysate of the cell types used, and the assays are proportional to time for at least 50 min. One unit of HGPRT activity is defined as the amount converting 1 μmol of hypoxanthine to inosinate per h. Conditions for isoelectric focusing and assay of HGPRT activity in acrylamide slab gels have been described (Johnson et al., 1979). Product formation in the assays of HGPRT activity in slab gels is proportional to the quantity of enzyme electrofocused (to a maximum of 52 milliunits of enzyme).

(*4*) *Density Fractionation of Red Cells.* Red cell populations with normal (1–3%) or high (6–18%) percentages of reticulocytes were fractionated according to cell age by the density gradient method of DeSimone et al. (1974). The interface and pelleted red cell populations of the step gradient were used as sources of the least dense (young) and the most dense (old) red cells for the linear gradients. The top or bottom fractions of the respective linear gradients were collected and rinsed as described by DeSimone et al. (1974). Fractionated cells were then rinsed and lysed by our method (Materials and Methods, 2). Reticulocytes were detected by using Wright's strain. Fewer than 0.3% nucleated cells contaminated the reticulocyte-rich fractions.

(*5*) *Enzyme Purifications.* (*I*) *Cell Lysates.* One unit of whole blood from each of 10 donors was collected in acid–citrate–dextrose. The freshly drawn blood was placed at room temperature for 2 h and the supernatant plasma removed. The cells were suspended in 4 volumes of buffer D and sedimented at 200*g* for 10 min. The supernatant fluid and white blood cells of the cell–fluid interface were discarded, and this rinsing procedure was repeated 4 times. Dithiothreitol was added to the packed cells to yield a concentration of 1 mM, and the cells were frozen in a dry ice–ethanol bath and stored at −80 °C. Frozen cells from 10 units of blood were thawed and mixed with 213 g (wet) of DEAE-cellulose (DE-52) equilibrated with 10 mM phosphate (K), pH 7.9, with 1 mM DTT. This mixture was stirred for 60 min and filtered through Whatman No. 4 paper. The filtrate fraction is designated the red cell lysate (Table II).

Human lymphoblasts were grown at 37 °C in a 5% CO_2 atmosphere in a medium consisting of 85% RPMI 1640 with 15% fetal calf serum. Cells were collected by centrifugation when the cell culture density reached (1–2) × 10^6 cells/mL. The cells were suspended in approximately 20 volumes of buffer D and collected by centrifugation at 500*g* for 10 min. This procedure for rinsing cells was repeated 4 times, and the rinsed cell pellets were frozen and stored at −70 °C. A total of 511 g of lymphoblasts (wet weight) was pooled, diluted to 1500 mL with buffer E, and frozen and thawed 4 times, to constitute the lymphoblast lysate fraction. This lysate was clarified by centrifugation at 500*g* for 15 min, yielding an S-0.5 fraction, and that supernatant fraction was sedimented at 100000*g* for 2 h to obtain an S-100 supernatant fraction. The materials pelleted in these centrifugations were discarded.

(*II*) *Batchwise Fractionation on DEAE-Sephacel.* All subsequent enzyme fractionations were carried out at 0–4 °C unless otherwise specified. The red cell lysate, 2640 mL, was diluted to 12 L with buffer E and mixed with 400 g (wet) of DEAE-Sephacel equilibrated in buffer F. The lymphoblast S-100 fraction, 1220 mL, was diluted with buffer E to 2.5 L and mixed with 100 g (wet) of DEAE-Sephacel equilibrated in buffer F. The extracts were mixed and stirred with the DEAE-Sephacel for 90 min, and the DEAE-Sephacel was collected by filtration through Whatman No. 4 filter paper. The DEAE-Sephacel was rinsed with 2 L of buffer H, and the filtrates were discarded. The DEAE-Sephacel was then suspended and stirred in 800 (red cell) or 500 mL (lymphoblast) of buffer H with 0.2 M NaCl for 30 min for elution of HGPRT. The filtrates (Whatman No. 4 filter paper) from two sequential extractions of the DEAE-Sephacel were pooled.

(*III*) *Ammonium Sulfate Fractionation.* The DEAE-Sephacel eluates were brought to 40% saturation by the addition of 242 mg of solid ammonium sulfate per mL (Schwarz/Mann, enzyme grade). The precipitate was separated by centrifugation at 15000*g* for 30 min and discarded. The supernatant was brought to 0.7 saturation by the addition of 204 mg of solid ammonium sulfate per mL. The precipitate was collected by centrifugation, and the supernatant was discarded. The pellet was dissolved in 100 (red cell) or 60 mL (lymphoblast) of buffer G and dialyzed against buffer G.

(*IV*) *Ethanol Precipitation.* The dialyzed fractions were brought to 0.25 M NaCl, and HGPRT was precipitated by the addition of 2 volumes of ethanol (dropwise with stirring). After 15 min, the precipitate was collected by centrifugation at 1000*g* for 15 min and the supernatant discarded. The pellet was extracted with 150 (red cell) or 80 mL (lymphoblast) of buffer H for 30 min at 37 (red cell) or 4 °C (lymphoblast) and recentrifuged. The extraction was repeated twice, and the three extracts were pooled.

(*V*) *Heat Treatment.* The soluble extract of the ethanol precipitate was brought to 5 mM $MgCl_2$, 1 mM P-Rib-PP, and 1 mM dithiothreitol and warmed to 37 °C, then heated to 85 °C for 15 min, and cooled in ice. The precipitated material was removed by successive centrifugations at 1500*g* for 5 min and 100000*g* for 60 min and discarded.

(*VI*) *Ammonium Sulfate.* The supernatant fraction from the preceding step was brought to 70% saturation by addition of 472 mg of solid $(NH_4)_2SO_4$ per mL, and the precipitate was collected by centrifugation at 25000*g* for 10 min. The precipitate was dissolved in 1 mL of buffer I with stirring for 60 min, and the insoluble material was removed by centrifugation at 40000*g* for 30 min. The solution was dialyzed overnight against buffer I.

(*VII*) *Sucrose Gradient Sedimentation.* The dialyzed ammonium sulfate fraction (0.4 mL/gradient) was centrifuged through a 10.5-mL linear 5–20% sucrose gradient in buffer I in an SW41 rotor (Beckman) at 40 000 rpm (286000g_{max}) for 48 h at 2 °C. Fractions were collected from the bottom of each tube and were stored at −70 °C.

(*6*) *Iodo[2-^{14}C]acetamide Labeling of HGPRT.* HGPRT from pooled sucrose gradient fractions was precipitated with 2 volumes of ethanol and the precipitate collected by centrifugation at 40000*g* for 30 min. The supernatant liquid was discarded, and the precipitate, containing 25–125 μg of protein, was dissolved in 0.05 mL of saturated guanidine hydrochloride in 0.5 M Tris-HCl, pH 8.2, and 10 mM EDTA with 8 mM dithiothreitol. Following room temperature incubation for 90 min, 5 μL of 0.11 M iodo[2-^{14}C]acetamide (New England Nuclear; 14 μCi/μmol) was added, and incubation was continued in the dark for 45 min. The labeled protein was dialyzed overnight against buffer J. The protein was then precipitated with 20% CCl_3COOH (2 times) and 2.5 mL of 1 N HCl (2 times), and the final pellet was dissolved in buffer J.

(*7*) *Acrylamide Gel Electrophoresis.* $NaDodSO_4$–acrylamide gel electrophoresis was performed as described by Laemmli (1970). The gels were stained for 15 min with 0.2% Coomassie blue in acetic acid–methanol–H_2O (7:45:48) and destained in acetic acid–methanol–H_2O (7.5:10:82.5). Protein

Table I: Relative Quantities of Activity in HGPRT Isoenzymes in Younger and Older Red Cell Populations[a]

	reticulocytes (%)	total HGPRT act. (%) in isoenzyme I	II	III	IV
(A) normal					
total	2 (1.3-3)[b]	25 (22-26)	33 (33-35)	32 (30-33)	10 (9-13)
young	13 (8.4-16.9)	47 (41-54)	33 (31-37)	18 (12-22)	2 (0-4)
old	0.4 (0.2-0.7)	20 (16-22)	36 (34-38)	35 (33-38)	9 (7-10)
(B) reticulocyte rich					
1. total	17	53	35	12	
young	39	71	24	4	
2. total	6	38	37	25	
young	16	52	35	12	
3. total	18	55	30	15	
young	59	64	26	10	
4. total	10	44	37	20	
young	36	61	29	10	
5. total	14	45	39	15	
young	93	74	22	4	

[a] The HGPRT enzyme of red cells was assayed in situ in slab gels with [8-^{14}C]hypoxanthine as substrate following electrofocusing (Materials and Methods). The relative quantities of activity in the isoenzymes I–IV were obtained by peak height analysis of densitometer scans of autoradiograms. Samples of individuals with normal (part A) or high (part B) reticulocyte percentages were analyzed before (total) and after density fractionation to obtain the least dense (young) and most dense (old) red cells (Materials and Methods). Red cell samples from five individuals were analyzed in part A. The values reported are averages, with the ranges for the five samples within parentheses. Isoenzyme IV was rarely detectable in the reticulocyte-rich samples (part B) and was not included in calculations of percent activity. [b] Range, $N = 5$.

distribution in the gel was estimated by absorbance measurements at 750 nm.

Isoelectrofocusing slab gels were prepared, and the enzyme was electrofocused and assayed for enzyme activity as previously described (Johnson et al., 1979). Gels containing 8 M urea were polymerized and electrofocused in the same way as the standard gels. Urea for these gels was deionized with a mixed bed resin (AG-50-1X8) (Bio-Rad). The protein distribution in the isoelectric focusing gels was determined after the gels were extracted at least 6 times with 500 mL of 5% CCl_3COOH to remove ampholines. These gels were stained for protein as described for the $NaDodSO_4$–acrylamide gels (see above). Iodo[2-^{14}C]acetamide-labeled proteins were detected by autoradiography.

(8) HGPRT Subunit Separation. The purifed red cell and lymphoblast enzymes were denatured and labeled with iodo-[2-^{14}C]acetamide (Materials and Methods, 6). Approximately 125 μg of each enzyme was then subjected to electrofocusing in 8 M urea (Materials and Methods, 7). The area of the gel containing the major subunit bands was sliced at 0.33-cm intervals, and each gel piece (0.33 × 1.2 × 0.416 cm) was placed in 1 mL of buffer J. The gel was extracted for 6 h at room temperature and then stored at −70 °C for 1 h. After thawing and separation from the gel, 250 μg of crystalline bovine serum albumin was added to the extract and the solution brought to 10% CCl_3COOH. The protein was collected by centrifugation at 12000g for 5 min. The protein precipitate was rinsed with 1 N HCl, dissolved in buffer J, and stored at −70 °C. Net recovery of labeled HGPRT protein in the extracts was approximately 35% of that applied to the gel.

(9) Trypsin Digestion and Peptide Chromatography. Iodo[2-^{14}C]acetamide-labeled HGPRT (25 μg; 2500 cpm/μg) in 0.01 mL of buffer J was mixed with 8 mg of human hemoglobin in 50 mM $NaHCO_3$ and digested with 400 μg of trypsin (TPCK treated, Worthington). Insoluble material was removed from the digests by centrifugation at 10000g for 10 min, and the tryptic peptides were separated by electrophoresis at pH 3.5 (Ingram, 1958). The area of the electrophoresis strip containing radioactive spots was cut out, sewn to a second sheet of paper, and chromatographed in the second dimension (butanol–pyridine–acetic acid–H_2O, 90:60:18:72) (Swenson et al., 1962). Globin peptides were detected by ninhydrin and labeled HGPRT peptides by autoradiography.

(10) Absorbance. Absorbance spectra were obtained with a Cary 118 spectrophotometer. HGPRT from peak sucrose gradient fractions (fraction VII) was precipitated with 2 volumes of ethanol and collected by sedimentation at 40000g for 10 min. The enzyme was dissolved in 1 mM phosphate (K), pH 6.8, with 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.25 M NaCl, at a concentration of approximately 0.25 mg/mL, and dialyzed to that solvent.

(11) Protein Determination. Protein concentrations were estimated as described by Lowry et al. (1951) with crystalline bovine serum albumin as a standard. Protein in fractions prior to DEAE-Sephacel chromatography was collected by precipitation in 10% CCl_3COOH prior to assay.

Results

(1) Separation of Red Cell HGPRT Isoenzymes. The isoelectric focusing pattern of HGPRT of circulating red cells and of lymphoblasts is shown in Figure 1a. The lymphoblast enzyme focuses at pH 6.30, while four species of the red cell enzyme focus at pH 6.30 (I), 6.16 (II), 6.05 (III), and 5.95 (IV) (see Figure 1 legend). The relative quantities of activity in the red cell isoenzymes, estimated by peak height analysis of densitometer tracings of this autoradiogram (Figure 1b), are 22% (I), 36% (II), 34% (III), and 8% (IV) (Figure 1b) (and see Table I). This report deals only with the properties of the major isoenzymes, I–III, because the estimated quantity of activity in isoenzyme IV is low and variable (5–10%). Two observations indicate that the red cell isoenzymes result from differences in the HGPRT protein charge rather than from interactions with the enzyme cofactor or other factors in red cell lysates. First, the focusing patterns of the red cell and lymphoblast enzymes do not change after preincubations during which enzyme–Mg–P-Rib-PP complexes are formed. Second, the single isoelectric species of the lymphoblast enzyme is not altered when preincubated in the presence of a red cell lysate lacking HGPRT activity (data not shown). Additional studies that provide evidence that the red cell isoenzymes do not result from changes that occur in vitro will be reported elsewhere (G. G. Johnson, unpublished results).

(2) Red Cell Age and Isoenzyme Content. We determined the distribution of activity among red cell isoenzymes from

Table II: Purification of HGPRT from Human Red Blood Cells and Lymphoblasts[a]

fraction	red cell enzyme			lymphoblast enzyme		
	vol (mL)	act. (units/mL)	sp act. (units/mg)	vol (mL)	act. (units/mL)	sp act. (units/mg)
I. lysate (S-100)	2640	17.7	0.074	1500 (1220)	12.35 (13.2)	0.77 (1.80)
II. DEAE-Sephacel	1280	19.5	1.9	800	23.4	4.8
III. 40–70% $(NH_4)_2SO_4$	152	161	3.1	83	101	3.1
IV. 0–66% ethanol	600	29.1	6.3	161	24.7	≥62
V. heated supernatant	600	24.7	180	340	19.1	ND
VI. 0–70% $(NH_4)_2SO_4$	1.4	6917	242	1.4	4052	155
VII. sucrose gradient	8.4	1099	1747	8	778	1410

[a] The details of fractionation of the enzymes were described (Materials and Methods). Because only 0.4 mL of the enzyme in fraction VI is centrifuged in a single sucrose gradient, the final step in purification uses centrifugation in parallel gradients. The estimates of HGPRT in fraction VII are based on one of these gradients and assume equal fractionation in the parallel gradients. ND, not determined.

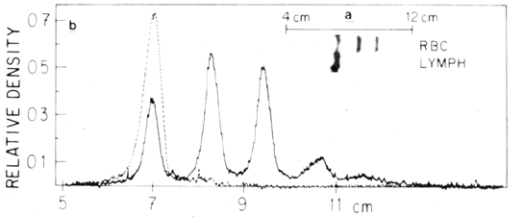

FIGURE 1: Electrofocusing of red cell and lymphoblast HGPRT activity. Sample preparation, electrofocusing, and assay of activity using [8-^{14}C]hypoxanthine as substrate are described under Materials and Methods. The inset figure (a) is a photograph of the autoradiogram of the red cell (RBC) and lymphoblast (LYMPH) enzymes electrofocused as adjacent samples. The 4–12-cm segment of the 20-cm gel is shown. Isoenzyme IV is not evident in this photographic reproduction. A densitometer scan of this autoradiogram is shown as part b: red cell (—); lymphoblast (- - -). The pH of isoelectric focusing of the four red cell isoenzymes is reported as the following: I, 6.30; II, 6.16; III, 6.05; IV, 5.95. These pH values are averages of eight separate determinations, with the range of values for isoenzyme I being pH 6.48–6.10. We estimate the pH values to be within ±0.1 pH unit of the isoelectric pH of these isoenzymes in this solvent. In contrast, the isoelectric focusing pH differences of the four isoenzymes are essentially constant, and we therefore estimate that isoelectric pH differences are within ±0.1 pH unit.

individuals with normal or with unusually high proportions of circulating reticulocytes, both before and after fractionation of the red cells according to cell age (Materials and Methods). In samples from individuals with normal numbers of reticulocytes, the "younger" red cells are enriched in isoenzyme I, while the "older" red cells are enriched in isoenzymes II–IV (Table IA). When compared with the unfractionated total red cell samples, in which 25% of the activity is in isoenzyme I, the younger and older populations of red cells of persons with normal red cell life span have approximately 47% and 20% of the enzyme activity as isoenzyme I, respectively. Further evidence of the change in red cell isoenzyme content with cell age was obtained by using samples from individuals with higher initial percentages of young cells (6–18% reticulocytes), which yielded samples with up to 93% reticulocytes when fractionated according to age. These more highly reticulocyte-enriched fractions have 38–74% of the enzyme activity as isoenzyme I (Table IB). Thus, isoenzyme I is seen to constitute up to 75% of the HGPRT activity in samples most highly enriched for reticulocytes. Isoenzymes II and III, on the other hand, represent an increasing fraction of the activity in the older red cells.

(*3*) *Purification of HGPRT.* Our purification method incorporates several steps previously used by others to purify the human red cell HGPRT enzyme (e.g., Krenitsky & Papaioannou, 1969; Olsen & Milman, 1974a; Gulumian & Wakid, 1975), although the details of fractionation differ substantially in two regards (a quantitative summary of the purification results is presented in Table II). First, fractionation on DEAE-Sephacel is batchwise with step elution (fraction II, Table II). The red cell isoenzymes can be partially separated by anion-exchange chromatography, and thus pooling of peak fractions, which can lead to selective loss of isoenzymes with different charges, is avoided. Second, the final step in purification is zone sedimentation in a solvent in which HGPRT sediments as a tetramer. This effectively removes lower molecular weight proteins that survive the heat regimen (step V), and HGPRT is recovered in a solvent in which it is stable (no activity loss detected on storage for 2 years at −70 °C).

The peak sucrose gradient fractions (fraction VII) for the red cell enzyme have a maximal specific activity of 1750 units/mg, and this is constant across the enzyme peak fractions (data not shown). The purification of the red cell enzyme (23 000-fold) is equal to that for the most highly purified preparation reported (Holden & Kelley, 1978). The sucrose gradient peak fractions for the lymphoblast enzyme have a maximal specific activity near 1400 units/mg which may be due to impurities in the peak fractions (although impurities are not clearly demonstrable; see later).

The major protein species in the purified preparations have indistinguishable subunit molecular weights, estimated to be 25 000–26 000 (Figure 2). This value is in agreement with reports for the subunit molecular weight of HGPRT isolated from human and mammalian cells (Olsen & Milman, 1974b, 1977; Hughes et al., 1975; Holden & Kelley, 1978).

(*4*) *HGPRT Isoenzymes.* The purified lymphoblast enzyme electrofocuses as a single species, isoenzyme I, while the three major red cell isoenzymes, I–III, are recovered in the purified preparation in relative quantities that approximate those in cell lysates.

We have also obtained evidence of a concordance between the distribution of HGPRT activity and the major protein species in the purified enzyme preparations in these native isoelectric focusing gels. The preliminary results from such studies indicate that they are complicated by the equilibrium between dimers and tetramers of the enzyme. For resolution and assay of the isoenzymes from cell lysates, we use 1–50 milliunits of HGPRT activity in 50 μL, which corresponds to an enzyme protein concentration of 10–500 ng/mL. At these concentrations, the enzyme electrofocuses as a dimer (Johnson et al., 1979). We have noted, however, that as the enzyme protein concentration is increased approximately 1000-fold to achieve sufficient quantities for protein analysis (e.g., 10 μg in 50 μL or 200 μg/mL), there is a shift in the isoelectric point of the HGPRT lymphoblast isoenzyme I from pH 6.3 to approximately pH 6.45 (at this higher concentration, the major protein species in the purified lymphoblast enzyme preparation electrofocuses at the higher pH value, and we obtain evidence of a concordance between enzyme activity and protein dis-

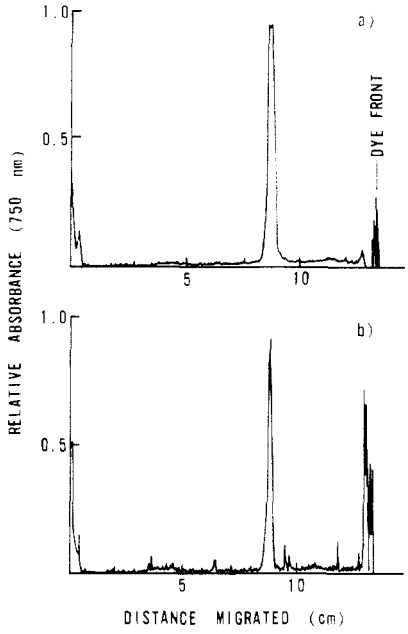

FIGURE 2: $NaDodSO_4$–acrylamide gel electrophoresis of HGPRT from red blood cells and lymphoblasts. Approximately 10 μg of the purified HGPRT enzymes (fraction VII, Table II) was electrophoresed in 10% acrylamide gels as described by Laemmli (1970). The gels were stained, and the protein was detected by absorbance measurements at 750 nm (Materials and Methods). The protein subunits from these cells electrophorese at equivalent rates, slightly slower than chymotrypsinogen A, when analyzed in adjacent slots in slab gels (data not shown). The samples analyzed were from the purified red cell (a) and lymphoblast (b) enzymes.

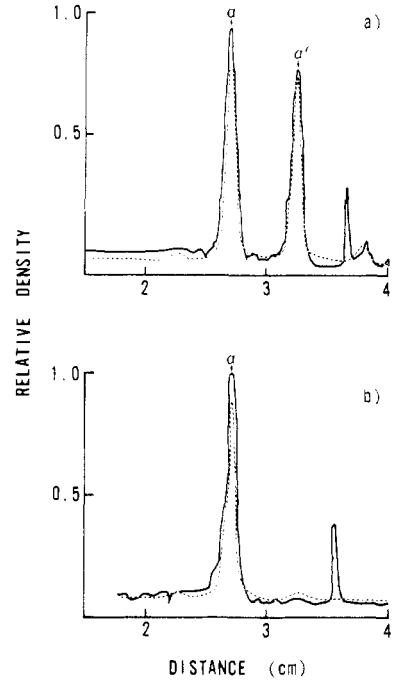

FIGURE 3: Isoelectric focusing of HGPRT in 8 M urea. The gel contained 2% pH 4–6 and 0.5% pH 3.5–10 ampholytes (LKB) and 8 M urea but was otherwise prepared and electrofocused as described (Materials and Methods). Purified HGPRT (fraction VII, Table II) was labeled with iodo[2-^{14}C]acetamide and electrofocused following acid precipitation (Materials and Methods). Protein was detected by staining (—) and autoradiography (---). Only the area of the gel containing protein or radioactively labeled protein bands is shown. The enzymes from red blood cells (a) and lymphoblasts (b) were analyzed in adjacent slots.

tribution). We tentatively attribute this shift at high enzyme concentrations to the formation of tetramers. Because of the concentration dependence of isoelectric focusing of HGPRT and because of the more complex pattern expected (and observed) for the red cell isoenzymes, which can form hetero-tetramers, we have not pursued these studies further in slab gels.

(5) *HGPRT Subunits.* The purified lymphoblast enzyme dissociates to yield a single subunit when electrofocused in 8 M urea (α) (Figure 3). The red cell isoenzymes dissociate into two subunits, one equivalent to that in the lymphoblasts (α) and one more negatively charged (α'). This result of obtaining a single HGPRT subunit from cultured cells and two from erythrocytes parallels that for the enzyme subunits isolated from these cell lysates by immunoprecipitation (Ghangas & Milman, 1977).

For comparisons of the tryptic peptide maps of the HGPRT subunits, the proteins were labeled with iodo[2-^{14}C]acetamide which is bound covalently to cysteine residues (Materials and Methods). (The labeled subunits have the same isoelectric points as the subunits not incubated with iodoacetamide, as expected in the absence of nonspecific reactions.) Tryptic peptide maps of the iodo[2-^{14}C]acetamide-labeled lymphoblast and red cell enzymes are shown in Figure 4. The digest of the lymphoblast enzyme yields a peptide map with five major labeled spots (Figure 4B). The digest of the red cell enzyme with the two subunits yields an identical map, with the exception of one additional peptide (Figure 4A). A composite drawing of the peptide maps is presented in Figure 4C and includes the relative yields in counts per minute in the peptides in the two digests. This result provides further evidence for the identity of the purified protein from these cells but also suggests that the unmodified and modified subunits differ at only one of the cysteine-containing tryptic peptides (labeled A and A′ in the drawing). This was confirmed by comparing

Table III: Recovery of A and A′ Peptides in HGPRT Subunits[a]

enzyme	subunit	fraction of label in peptide A	A′
lymphoblast	α	0.99	0.01
red cell	α	0.94	0.06
red cell	α'	0.13	0.87

[a] HGPRT was labeled with iodo[2-^{14}C]acetamide, and the subunits were isolated by isoelectric focusing as described (Materials and Methods; Figure 3). Tryptic peptides of the subunits were obtained and separated as described (Materials and Methods; Figure 4). The radioactively labeled peptides were detected by autoradiography and quantitated by scintillation counting in a Liquifluor-toluene solvent. The locations of the A and A′ peptides in digests of HGPRT are shown in Figure 4. The recovery of label in the A and A′ peptides is expressed as a fraction of the total counts per minute recovered in these two peptides in the individual digests. The net counts per minute recovered in the two peptides in the digests of the lymphoblast (α) and the unmodified (α) and modified (α') red cell enzyme subunits were 3542, 1179, and 1003 cpm, respectively. See text and Figure 3 for subunit designations.

the tryptic peptide maps of the single lymphoblast (α) and two red cell subunits (α and α') following separation by isoelectric focusing (Materials and Methods). Digests of the lymphoblast and one red cell subunit (α) yield peptide A almost exclusively, while the modified red cell subunit (α') yields peptide A′ almost exclusively (Table III). These results do not, of course, exclude the possibility that there are other modifications or differences between the subunits in these cells (which are estimated to contain approximately 30 tryptic peptides; Olsen & Milman, 1977).

(6) *Absorbance Spectra.* The absorbance spectra of the purified proteins were determined over the range of 230–300 nm (Materials and Methods). The absorbance maxima and

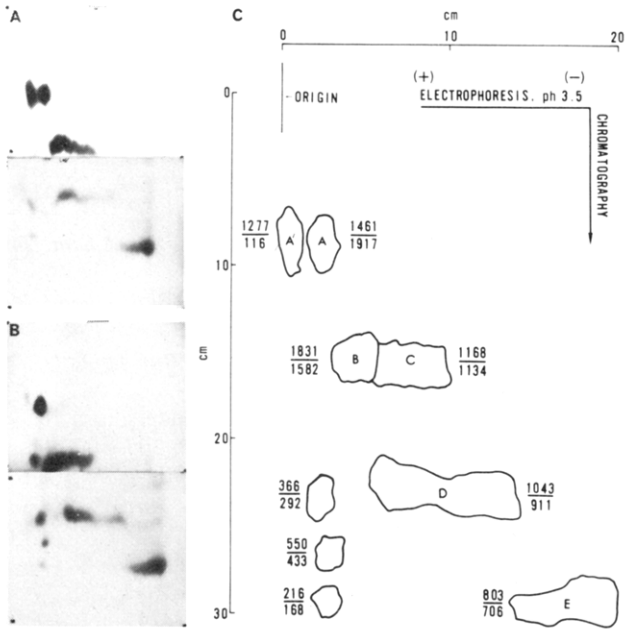

FIGURE 4: Tryptic peptide maps of iodo[2-^{14}C]acetamide-labeled HGPRT. The HGPRT proteins from red cells and lymphoblasts were labeled with iodo[2-^{14}C]acetamide and digested with trypsin (Materials and Methods). Each digest contained 8 mg of unlabeled human α- and β-globin protein and approximately 25 μg of HGPRT (2500 cpm/μg). A core of undigested protein was removed by sedimentation at 10000g. Approximately 15% of the radioactively labeled protein was removed along with this core in digests of both enzymes. The tryptic peptides were separated by electrophoresis at pH 3.5 followed by chromatography in the second dimension (Materials and Methods). The α- and β-globin peptides were detected by ninhydrin and iodo-[2-^{14}C]acetamide-labeled HGPRT peptides by autoradiography. (A) Red blood cell enzyme. (B) Lymphoblast enzyme. (C) A composite map of the radioactively labeled peptides in the two digests, with the five major peptides designated A–E. Our estimate from incorporation of iodo[2-^{14}C]acetamide to protein and those of others on the amino acid composition of HGPRT indicate that the subunits contain four to five cysteine residues (Olsen & Milman, 1977). The single additional major peptide in the digest of the red cell enzyme is labeled A′ and is presumably a modified form of the A peptide (see text and Table III). The recovery of label in the five major tryptic peptides was determined by scintillation counting and is indicated by the number adjacent to the peptide, with the counts per minute in digests of the red cell enzyme over those for the lymphoblast enzyme. The net recovery of label in the major peptides was 40% of that added to the digests. The three peptides in the lower left corner of the map were recovered in digests of both enzymes and are presumably due to incomplete trypsin digestion or chymotryptic cleavages. They are also present in tryptic digest of the red cell and lymphoblast subunits that have been further purified by isoelectric focusing in urea (data not shown). (The horizontal line through the center of the figures is at the point of contact of two pieces of X-ray film layered over the single chromatographic sheet.)

spectra were virtually identical and provided estimates of $A_{280}^{1\%}$ of 8.5 for total HGPRT protein and a molar extinction coefficient for the subunit at 260 nm of 15000 M^{-1} cm^{-1} (data not shown). Considering that approximately one-half of the HGPRT subunits in red cells are modified (Results, 5), the virtual equivalence in the absorbances and spectra for the proteins purified from red cells and lymphoblasts argues against the possibility that the modification of the subunit in red cells is by covalent addition of a nucleotide (e.g., adenylylation).

Discussion

Our criteria for purity of the HGPRT enzyme from human lymphoblasts include subunit homogeneity by molecular weight in $NaDodSO_4$ and by charge in urea. The single subunit (α) indicates that the enzyme is a homo dimer ($\alpha\alpha$). This is consistent with results of the studies of HGPRT isolated by immunoprecipitation from human (HeLa) and mouse (L) cells in culture and with genetic evidence of a single HGPRT structural gene in the mammalian X chromosome (Seegmiller et al., 1967; Wahl et al., 1975; Chapman & Shows, 1976; Ghangas & Milman, 1977).

The purified lymphoblast enzyme served as a standard for identifying the three major HGPRT isoenzymes in human erythrocytes. The present evidence indicates that the three isoenzymes are related to the occurrence of two HGPRT subunits, one equivalent to that in lymphoblasts (α) and one more negatively charged (α'). The simplest interpretation is that the enzyme in young red cells is identical with the enzyme in lymphoblasts but that the former is modified in the erythroid cells. HGPRT electrofocuses as a dimer, and the three identified isoenzymes would correspond to enzyme species with none ($\alpha\alpha$, isoenzyme I), one ($\alpha\alpha'$, isoenzyme II), or two ($\alpha'\alpha'$, isoenzyme III) modified subunits.

The evidence that the α' subunit results from posttranslational change of the α subunit is first suggested by their identity for four of five cysteine-containing tryptic peptides. We have shown that the greatest changes in relative abundances of the isoenzymes occur in the enucleated reticulocyte or later in the red cell life, and it is unlikely that II and III increase as a result of de novo synthesis. This interpretation is also supported by the evidence from studies of red cells from individuals with the Lesch–Nyhan syndrome. The individuals lack HGPRT activity in all cells tested (Rosenbloom et al., 1967b) and lack all three of the isoenzymes of red cells.

The results indicate that the HGPRT enzyme in red cells is subject to at least one site-specific (one cysteine-containing tryptic peptide), posttranslational alteration. We have compared only five tryptic peptides, and the α and α' subunits may differ at additional sites. At the same time, however, the increased negative charge in the altered peptide (A′; Figure 4) could explain the difference in charge between the α and α' subunits, and it may be the only difference. Alterations that either lower the net positive charge or add negative charge to the subunit could explain the change. The charge change is evident at pH 3.5, which rules out deamidation of glutamine.

This single modification of the HGPRT subunit provides only a partial accounting of the changes HGPRT undergoes in human erythroid cells. In addition to the major isoenzymes, I–III, lesser but significant quantitites of HGPRT are recovered in even more acidic isoenzymes (e.g., isoenzyme IV, Figure 1). The more acidic isoenzymes may result from further modifications of the HGPRT subunit [evidence of the existence of an additional modified form of the HGPRT subunit has been presented (Zannis et al., 1980)]. In studies to be reported elsewhere, we provide evidence that the levels of HGPRT activity and immunoreactive protein decrease with red cell age, indicating that the enzyme is degraded. The relationship between modification and degradation of HGPRT is, as yet, unclear.

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