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Purification and Characterization of Hypoxanthine–Guanine Phosphoribosyltransferase from *Saccharomyces cerevisiae*†

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ABSTRACT: Hypoxanthine–guanine phosphoribosyltransferase (HPRT) was purified 12 000-fold to homogeneity from yeast by a three-step procedure including acid precipitation, anion-exchange chromatography, and guanosine 5′-monophosphate affinity chromatography. The enzyme is a dimer consisting of two, probably identical, subunits of M_r 29 500. The enzyme recognizes hypoxanthine and guanine, but not adenine or xanthine, as substrates. An antiserum against both native and denatured enzyme has been raised and shown to be specific for the enzyme. The antiserum has no affinity for Chinese hamster or human HPRT but does recognize subunits of yeast HPRT as well as some cyanogen bromide fragments of the enzyme.

Hypoxanthine–guanine phosphoribosyltransferase[1] (HPRT; EC 2.4.2.8) catalyzes the conversion of hypoxanthine and guanine to their respective nucleotides. This enzyme from mammalian sources has proved to be of great interest for the study of mutation in cultured mammalian cells (Caskey & Kruh, 1979). Deficiency of the enzyme in humans causes

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[1] Abbreviations used: HPRT, hypoxanthine–guanine phosphoribosyltransferase; GMP, guanosine 5′-monophosphate; PRPP, 5-phosphoribosyl 1-pyrophosphate; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride.

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Lesch-Nyhan syndrome, and partial deficiencies cause gouty arthritis (Kelley & Wyngaarden, 1978). The analogous enzymatic activity in yeast cells has been far less extensively studied. Only one previous report of purification to homogeneity of this enzyme from yeast is in the literature (Schmidt et al., 1979) Few papers have addressed the question of substrate specificity and kinetic parameters (Miller & Bieber, 1968, 1969; Ali & Sloan, 1980). We have undertaken a study of this enzyme for two main purposes. First, we wished to purify the enzyme to homogeneity and characterize its structure and kinetic properties. Furthermore, we wished to obtain an antibody preparation specific for yeast HPRT and capable of recognizing enzyme fragments. Such antibody preparations can be used to evaluate the structural homologies between yeast and mammalian HPRTs and, furthermore, to detect either active HPRT or enzymatically inactive fragments of HPRT made by bacteria carrying plasmids containing cloned pieces of yeast DNA. We report the purification and characterization of HPRT from *S. cerevisiae* and the characterization of an antiserum raised agianst the enzyme.

Experimental Procedures

HPRT Purification. HPRT activity was determined at 37 °C as previously described by use of [^{14}C]hypoxanthine (Fenwick & Caskey, 1975). One unit of HPRT catalyzes formation of 1 nmol of inosine 5′-monophosphate (IMP) min^{-1}. Protein was measured by the method of Lowry (Lowry et al., 1951).

(*A*) Purification steps were carried out at 4 °C. One-half pound of baker's yeast (Red Star) was suspended in buffer A (25 mM Tris-Cl, pH 7.8, 20 mM KCl, 6 mM $MgCl_2$, and 1 mM dithiothreitol); the final volume was 350 mL. The suspension was made 1 mM in phenylmethanesulfonyl fluoride (PMSF) and 50 mM in benzamidine by the addition of an aliquot of 40 mM PMSF in 95% ethanol and of solid benzamidine hydrochloride. The cells were disrupted by five passes through a Gaulin press at 3000 psi. The temperature was maintained between 4 and 8 °C during cell disruption. The lysate was centrifuged at 10000*g* for 30 min, and the supernatant (fraction I, 350 mL, 71 mg/mL protein) was removed free of cell debris.

(*B*) Fraction I was acid precipitated by dropwise addition of glacial acetic acid to a pH of 4.5. After being stirred for 15 min, the suspension was clarified by centrifugation (10000*g* for 30 min) and the supernatant was removed (fraction II, 300 mL, 30 mg/mL protein). Tris base (2 M) was added to adjust the pH to 7.8.

(*C*) Fraction II was dialyzed for 18 h against 10 volumes of buffer B (25 mM Tris-Cl, pH 7.8, 20 mM KCl, 6 mM $MgCl_2$, 1 mM dithiotreitol, and 50 mM benzamidine) with one change. A flocculent precipitate in the dialysis bag was removed by centrifuguation. Fraction II (520 mL) was applied to a DEAE-Sephadex A-50 column (2.6 × 40 cm), equilibrated in buffer B, at a flow rate of 50 mL/h; 20-mL fractions were eluted with 540 mL of buffer B followed by a 1100-mL gradient containing linearly increasing KCl from 20 to 500 mM in buffer B. The fractions containing activity (Figure 1A, tubes 62–81) were pooled and concentrated by pressure filtration using an Amicon PM10 membrane. This partially purified preparation (fraction III, 72 mL, 2.9 mg/mL) could be stored in aliquots at −70 °C for 20 days without detectable loss of activity.

(*D*) A GMP affinity column (1.4 × 50 cm) was prepared with guanosine 5′-monophosphate linked by 3,3′-iminobis-(propylamine) to Sepharose 4B (Hughes et al., 1975). A 10-mL aliquot of fraction III was dialyzed against 10 volumes

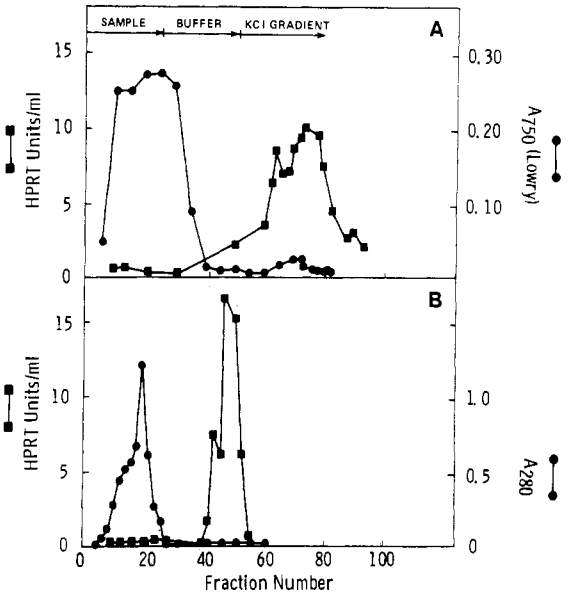

FIGURE 1: DEAE-Sephadex A-50 chromatography of fraction II. Protein (●) measured by absorbance at 750 nm (Lowry et al., 1951); HPRT (■) in units/mL. GMP-Sepharose chromatography of fraction III. Protein (●) measured by absorbance at 280 nm; HPRT (■) in units/mL.

of buffer C (50 mM Tris-Cl, pH 7.4, 25 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT) for 18 h with one change prior to affinity column application. Following application of fraction III and 3 mL of buffer C, the flow was halted for 20 min to allow equilibration of HPRT with the affinity matrix. Fractions devoid of HPRT were eluted by 165 mL of buffer C at a flow rate of 20 mL/h. HPRT was eluted by 1 mM 5-phosphoribosyl 1-pyrophosphate (PRPP) in buffer C. The fractions containing activity (Figure 1B, tubes 42–52) were pooled, concentrated by pressure filtration (fraction IV) as described above, and stored in buffer C with 1 mM PRPP at −120 °C. The GMP affinity column was reused for as many as ten successive preparations. Following use it was washed with 200 mL of 6 M urea and 200 mL of buffer C made 1.5 M in KCl and 0.2% in NaN_3 (w/v) and stored in this buffer at 4 °C.

This purification procedure was carried out 5 times with five separate samples of commerical baker's yeast. An identical purification procedure was also carried out, on a smaller scale, with a laboratory strain of *S. cerevisiae*, X2180-1Aa, grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) to mid-log phase. The strain was obtained from the Yeast Genetic Stock Center, Berkeley, CA.

Molecular Weight Determination. (*A*) *Molecular Sieve Chromatography.* A 1.5 × 90 cm column of Sephadex G-100-150 was equilibrated in buffer C containing 0.1 mM Na_4PRPP and used at 4 °C for molecular weight estimation. The elution volume of 1000 units of HPRT (fraction IV) in 0.8 mL was determined employing a flow rate of 3 mL/h. The elution volume for HPRT was detected by enzymic assay. Bovine serum albumin and ovalbumin were measured by their absorbance at 280 nm and myoglobin was measured by its absorbance at 407 nm. Aliquots of column fractions were desalted by dialysis against 1000 volumes of 0.1 M NH_4HCO_3 and lyophilized. The lyophilized material was dissolved in sample buffer and applied to a 17.5% polyacrylamide slab gel for electrophoresis (polyacrylamide gel) with sodium dodecyl sulfate (Olson & Milman, 1974).

(*B*) *Equilibrium Sedimentation.* The molecular weight of yeast HPRT was determined by equilibrium sedimentation for 3 h at 22000 rpm at 10 °C in buffer C. Scanning optics were

used to measure absorbance at 280 nm. A solution of HPRT (fraction IV) containing 100 μg/mL was used. Molecular weight was estimated both by averaging the weights calculated from the absorbance at each of 20 points along the cell and by the slope of a straight line fit by least squares to a plot of R^2 vs. ln OD_{280}. The partial specific volume was assumed to be 0.74.

(C) Polyacrylamide Gel Electrophoresis with $NaDodSO_4$. Yeast HPRT (fraction IV, 2 μg) underwent electrophoresis through 1.5 mm thick slab gels containing 15% and 17.5% polyacrylamide in the presence of $NaDodSO_4$ as previously described (Olson & Milman, 1974). The gels were stained with 0.2% Coomassie blue in 50% methanol and 7.5% acetic acid, and the mobility of yeast HPRT was compared to that of bovine serum albumin, ovalbumin, triosephosphate isomerase, and myoglobin.

Substrate and Kinetic Studies. (A) Native Polyacrylamide Gel Electrophoresis. Yeast HPRT (fraction IV) underwent electrophoresis through a 0.6 × 6.4 cm 9% polyacrylamide tube gel at 4 °C using a 3.4% polyacrylamide stacking gel as previously described (Fenwick et al., 1977a). The gel was cut into 5-mm slices and equilibrated 12 h at 4 °C in 50 μl of buffer D (50 mM Tris, pH 7.4, and 5 mM $MgCl_2$). HPRT activity was detected by addition of ^3H- or ^{14}C-labeled purine base and PRPP to the gel and eluate followed by a 1-h incubation at 37 °C. Formation of ^{14}C- or ^3H-labeled nucleotides was detected on 25-μL aliquots as previously described (Fenwick & Caskey, 1975).

(B) Kinetic studies were carried out with fraction IV equilibrated in buffer E [50 mM Tris-Cl, pH 7.4, 5mM $MgCl_2$, 1 mg/mL albumin (BSA)]. Hypoxanthine, specific activity 16.6 Ci/mmol, was diluted with cold hypoxanthine to give a stock solution of 2.33×10^{-4} M hypoxanthine, specific activity 1.07 Ci/mmol. Reactions, in duplicate, were carried out for 5 min at 37 °C with 0.033 unit of HPRT (fraction IV) in 50 mL of 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1.5 mM Na_4PRPP, and 200 mg/mL BSA; hypoxanthine concentrations ranged from 3.1×10^{-5} to 7.8×10^{-6} M. Reactions were terminated by the addition of 10 μL of 0.5 M Na_2EDTA and duplicate 20-μL aliquots from each reaction were quantitated for [^3H]IMP formation (Bendek & Patel, 1977). For inhibitor studies, the inhibitor was present prior to enzyme addition. Activity is calculated as moles of IMP produced per liter per second. Data were analyzed by Linewater–Burk plots; straight lines were drawn by a least-squares fit procedure.

Preparation of Antibody. Twenty micrograms of fraction IV was denatured overnight at room temperature in 50% (w/v) guanidine hydrochloride in buffer C and then mixed with an equal volume of complete Freund's adjuvant. Twenty micrograms of native HPRT was also mixed with equal volumes of complete Freund's adjuvant. A yearling goat was immunized by injecting one half of the denatured and one half of the native antigen subcutaneously into two separate sites and the remaining half of each antigen intramuscularly into two more separate sites. The injections were repeated 2 weeks and 6 months later. Serum samples were obtained weekly for 4–6 weeks after each injection, and the antibody response was measured by immunoprecipitation of HPRT as follows. All sera were heated 56 °C for 1 h before use. Sera to be tested were diluted in preimmune serum, and 20 μL of each dilution was mixed with 20 μL of HPRT (fraction IV diluted to 15 μL/mL) in 1.5-μL polypropylene tubes for 1 h at 4 °C. After the addition of formalinized *Staphylococcus aureus* (The Enzyme Center), the tubes were held at 4 °C for 5 min and then centrifuged for 5 min in a microfuge. A 10-μL aliquot of supernatant was assayed for HPRT activity. Antibody against denatured HPRT was assayed by Ochterlony immunodiffusion in 1% agarose against HPRT that had been denatured in 50% (w/v) guanidine hydrochloride and then dialyzed extensively against buffer C. Ochterlony plates were developed for 1–2 days, washed extensively with phosphate-buffered saline, and stained with 0.2% Coomassie blue.

Table I: Purification of Yeast HPRT

fraction	step	total protein (mg)	sp act. (units/mg)	total act. (units)
I	extract	2.49×10^4	2.5	6.2×10^4
II	pH 4.5	1.26×10^4	5.0	6.3×10^4
III	DEAE-Sephadex	2.12×10^2	2×10^2	4.3×10^4
IV	affinity chromatography	1.27	3×10^4	3.8×10^4

An HPRT antigen column was prepared by binding 700 μg of purified HPRT to 1 mL of cyanogen bromide activated Sepharose 4B and was used to purify anti-HPRT (Palmiter et al., 1971). Approximately 80 μg of purified anti-HPRT could be obtained from each 7.5 mg of crude antiserum. Purified antibody was radiolabeled with ^{125}I using lactoperoxidase and glucose oxidase (Enzymobeads, Bio-Rad) to a level of $(1–2) \times 10^7$ cpm/μg (Hubbard & Cohn, 1972). Staph protein A was similarly labeled.

Antigenic Specificity of Antiserum. Yeast HPRT (1 μg) underwent cyanogen bromide cleavage, and the fragments were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis in 20% acrylamide with 0.2% bis(acrylamide). In parallel lanes molecular weight markers and 1 μg of uncleaved yeast HPRT also underwent electrophoresis. The peptides were then transferred electrophoretically to nitrocellulose filters (Towbin et al., 1979). Purified, radiolabeled antibody was incubated with one half of the filter and the location of antibody binding detected by exposing the filter to X-ray film for 5–7 days at −70 °C. The other half of the filter was stained for protein with 0.1% Amido Black. Purified Chinese hamster HPRT was also submitted to electrophoresis and then transferred to filters. Three pairs of lanes containing hamster (0.5 μg) and yeast (0.5 μg) HPRT were incubated with three different antisera: antiserum against native Chinese hamster HPRT (Fenwick, et al., 1977b), antiserum against denatured Chinese hamster HPRT (Caskey et al., 1979), and antiserum against yeast HPRT. The filters were then incubated with ^{125}I-labeled protein A and the location of antibody binding was determined. A fourth set of lanes containing molecular weight markers and the two HPRT antigens were stained for protein.

Results

Yeast HPRT was purifed 12000-fold (Table I) over the activity in crude extracts, using three successive steps. The overall recovery of activity was 61%, indicating the methods are highly efficient. As shown in Figure 1, GMP–Sepharose affinity chromatography provided the greatest purification. We found it necessary to partially purify the HPRT prior to affinity chromatography for optimal binding and maximum purification. Using such partially purified fractions we detected no loss in affinity column capacity over ten successive column usages. Capacity of the GMP–Sepharose affinity column was 500 units of HPRT per mL of Sepharose beads. These studies indicate affinity chromatography is an effective means of enzyme purification for HPRT when used in combination with conventional purification methods.

The application of these purification procedures yielded an HPRT preparation which was highly purified by a variety of

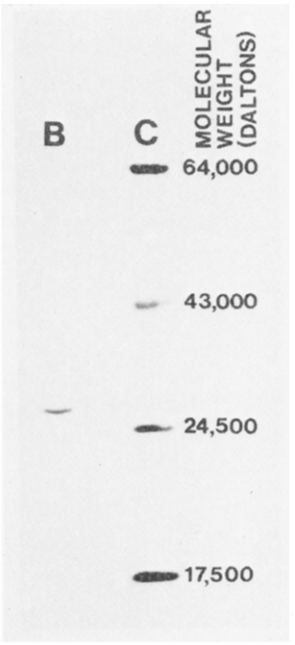

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis in 15% polyacrylamide. Lane B contains 2 μg of fraction IV stained with Coomassie blue. Standards in lane C are bovine serum albumin, ovalbumin, triosephosphate isomerase, and myoglobin, with molecular weights as shown.

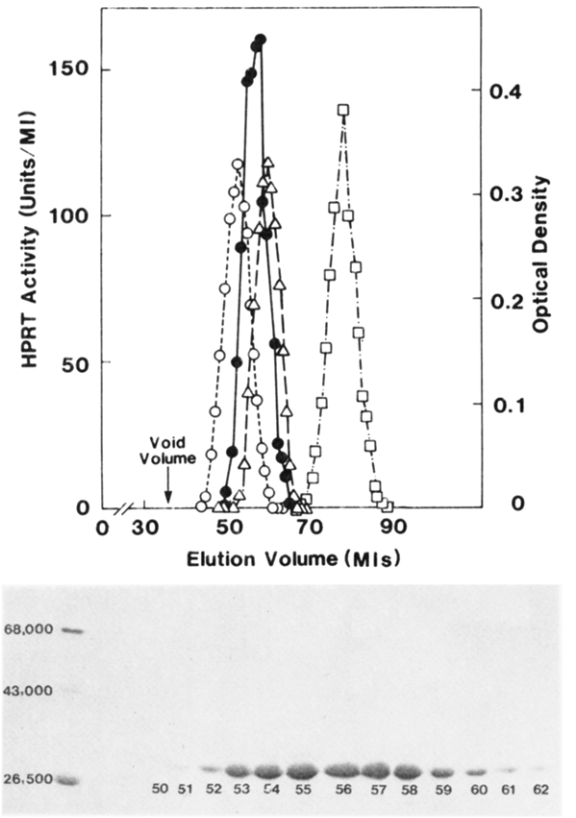

FIGURE 3: (Top) Sephadex G-100 chromatography of fraction IV. HPRT activity in units/mL (●) is plotted against fraction number. Bovine serum albumin (○) and ovalbumin (△) were detected by their absorbance at 280 nm; myoglobin (□) was detected by its absorbance at 407 nm and plotted as $A_{407}/10$. (Bottom) $NaDodSO_4$–polyacrylamide gel electrophoresis of fractions 50–62 in 17.5% polyacrylamide. Gel is stained with 0.2% Coomassie blue. Molecular weight markers are shown on the left, and the elution volume of each lane is shown at bottom.

criteria. The activity of our most purified fraction (Figure 2), when determined at a variety of enzyme levels, was 30 units/μg. This value compares favorably with the reported activity of human HPRT (Holden & Kelley, 1978). The substrate specificity and kinetic properties of the highly purified yeast HPRT have been determined by several methods. A single peak of activity with R_f 0.6 was identified when HPRT was subjected to polyacrylamide gel electrophoresis under nondenaturing conditions and either hypoxanthine or guanine was used as substrate (data not shown). A single protein, therefore, has guanine phosphoribosyltransferase (PRT) and hypoxanthine PRT activity. Adenine was not recognized by the HPRT. This conclusion was supported by kinetic studies which examined the recognition of a number of naturally occurring purines as well as the purine analogue 6-thioguanine. When [^{3}H]hypoxanthine was used as substrate, the concentrations of guanine and 6-thioguanine that reduced [^{3}H]IMP formation by 50% were determined over a range of hypoxanthine concentrations. Inhibition was competitive by Lineweaver–Burk plots. 6-Thioguanine had the lowest K_i, followed by guanine. Adenine and xanthine did not compete with hypoxanthine even at concentrations 10-fold higher than the K_m for hypoxanthine. The concentration of PRPP used was 100-fold higher than the published K_m values for this substrate and represents a saturating concentration for PRPP (Miller & Bieber, 1968).

The physical properties of yeast HPRT have been examined by several methods. Molecular weight estimates have been made by equilibrium sedimentation analysis, $NaDodSO_4$–polyacrylamide gel electrophoresis, and molecular sieve chromatography. As shown in Figure 2, a single protein with M_r 29 500 was detected by Coomassie blue staining of a denaturing ($NaDodSO_4$) polyacrylamide gel. Repeat preparations showed the same protein band migrating with the same molecular weight. Altering the percentage of acrylamide in the gel did not change the estimate of the subunit molecular weight. Equilibrium sedimentation analysis under native conditions gave a molecular weight of 54 800 as calculated from the slope of a linear regression line in the square of the radius vs. the natural log of the absorbancy at 280 nm. The point-average molecular weight was 59 200. The discrepancy in these two values is due to the nonlinearity that occurred at points at the bottom of the centrifuge cell. The HPRT molecular weight estimate by molecular sieve chromatography was 54 500 (Figure 3, top). When active fractions eluting from the Sephadex G-100 column were examined by $NaDodSO_4$–polyacrylamide gel electrophoresis, a single protein of M_r 29 500 was found (Figure 3, bottom). Protein staining correlated in intensity to the HPRT activity. On the basis of these studies, enzymically active yeast HPRT is a dimer composed of subunits of identical molecular weight.

The immunologic characteristics of yeast HPRT have been examined and compared to those of hamster HPRT. When an immunization procedure designed to give rise to antibodies to both native and denatured HPRT was used, goat anti-HPRT was obtained. As shown in Figure 4, the antibody was highly specific for HPRT since only a single broad immunoprecipitation line of identity was observed when highly purified HPRT (fraction IV, well 5) or partially purified HPRT (fraction III, well 4) was used. This serum recognized both native (well 3) and denatured (well 2) HPRT. The spur formation observed in the native HPRT precipitin line vs. the denatured HPRT precipitin line indicates some immunologic determinants recognized by the antibody are present only in the native enzyme. A similar immunoprecipitation pattern was seen when anti-HPRT purified on an HPRT antigen column was used in place of crude antiserum (data not shown).

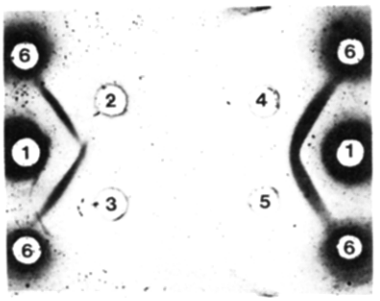

FIGURE 4: Ouchterlony immunodiffusion in 1% agarose. Well 1 contains antiyeast HPRT antiserum and well 6 contains preimmune serum. Wells 3 and 5 contain fraction IV, well 4 contains fraction III, and well 2 contains denatured fraction IV. Precipitation lines are stained with 0.2% Coomassie blue.

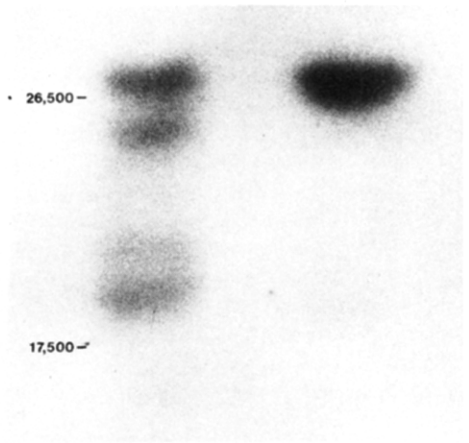

FIGURE 5: Autoradiography of nitrocellulose filter onto which yeast HPRT (right-hand lane) and cyanogen bromide digested yeast HPRT (left-hand lane) have been transferred electrophoretically from an $NaDodSO_4$–polyacrylamide gel. The filter was then incubated with ^{125}I-labeled purified antiyeast HPRT. The migration of triosephosphate isomerase and myoglobin is shown on the left-hand margin as determined from a duplicate nitrocellulose filter stained for protein with 0.1% Amido Black.

The recognition by this antiserum of native yeast HPRT was confirmed when immunoprecipitation followed by enzyme assay of the supernatant was used. The antibody immunoprecipitated all HPRT activity from fraction I and fraction IV (data not shown). The log of activity remaining was linear against concentration of antiserum. The antiserum failed to immunoprecipitate Chinese hamster or human HPRT from extracts of cultured cells (data not shown).

The recognition of denatured HPRT by this antiserum was confirmed by studies using purified HPRT transferred from gels to nitrocellulose filters after $NaDodSO_4$–polyacrylamide gel electrophoresis. As shown in Figure 5, in the right-hand lane, anti-HPRT (yeast) recognized a single protein of M_r 29 500. The antibody also recognized peptides resulting from partial cyanogen bromide digestion of yeast HPRT. As shown on the left-hand lane of Figure 5, three fragments ranging between M_r 25 000 and 18 000 bound radiolabeled purified anti-yeast HPRT. The antibody did not recognize Chinese hamster HPRT, however, and antibodies specific for native and denatured forms of Chinese hamster HPRT did not recognize yeast HPRT (data not shown). These studies suggest yeast and hamster HPRT differ significantly in structure.

Discussion

GMP affinity chromatography has been used for the purification of mammalian HPRT (Hughes et al., 1975; Holden & Kelley, 1978). Previous work with yeast HPRT has relied, for the most part, on partially purified preparations obtained through more conventional, less powerful, separation methods. Recently, however, Schmidt et al. have reported the use of GMP affinity chromatography for the purification of the enzyme from commercial baker's yeast (Schmidt et al., 1979). Whereas they used GMP linked through aminohexane to Sepharose and used 1.2 M KCl throughout the procedure, we used 3,3′-iminobis(propylamine) as a linker and did not use high salt concentrations during any stage of the purification. Overall, the two purification procedures give comparable results with 9400-fold and 12 000-fold purifications. The specific activities are difficult to compare because Schmidt et al. failed to define their units of activity. The yeast HPRT fraction we purified has a specific activity of 3×10^4 units/mg, similar to that reported for highly purified human HPRT (Holden & Kelley, 1978).

Limited information has been available on the structure of HPRT from yeast. A molecular weight estimate of 42 000–48 000 for HPRT from *Schizosaccharomyces pombe* was reported when controlled-pore glass chromatography (Nagy & Ribet, 1977) was used. We found a molecular weight estimation of 54 500 by Sephadex G-100 chromatography, and Schmidt et al. report a similar result of 51 000 by the same technique. A discrepancy does exist between the subunit molecular weight of 29 500 as determined by $NaDodSO_4$–polyacrylamide gel electrophoresis and the native molecular weight of 54 800 as determined by equilibrium sedimentation. $NaDodSO_4$–polyacrylamide gel electrophoresis is subject to variables that can confound accurate molecular weight determination (Nielsen & Reynolds, 1978). Glycosylated proteins migrate aberrantly in $NaDodSO_4$–polyacrylamide gel electrophoresis; yeast HPRT is not, however, heavily glycosylated (data unpublished). Yeast HPRT (100 μg), bovine serum albumin (an unglycosylated protein) (100 μg), and human transferrin (100 μg, 6% carbohydrate by weight) underwent electrophoresis with $NaDodSO_4$ in a 3-mm-thick polyacrylamide slab gel; after carbohydrate staining, transferrin alone stained darkly (Glossman & Neville, 1971). Albumin and yeast HPRT showed no staining. Even in the absence of glycosylation, however, molecular weight determination is relative to markers, and the migration of an unknown protein may deviate from the "true" expected migration because of differences in $NaDodSO_4$ binding or molecular conformation between the standards and unknown (Banker & Cotman, 1972). Equilibrium sedimentation is also subject to some variability. The plot of ln OD vs. R^2 gave, predominantly, a straight line, as one expects with a homogeneous protein that is behaving ideally. The molecular weight estimate, however, depends on the partial specific volume, a number which must be estimated when measurement is not possible. In our case, 0.74 was taken as a reasonable estimate. Data smoothing and least-squares regression line-fitting may obscure the true molecular weight in the face of nonlinearities in the sedimentation behavior (Aune, 1978). For this reason, point molecular weight was also examined as a check on the ideality of the protein undergoing sedimentation. The point molecular weight estimate for yeast HPRT was 59 200, a value in closer agreement with twice the $NaDodSO_4$–polyacrylamide gel electrophoresis subunit estimate. The point-average molecular weight exceeded that of the least-squares regression line value because of higher moecular weight estimates arising from points near the bottom of the centrifuge cells where protein interactions may have occurred at high protein concentrations. Molecular sieve chromatography is well-known to provide only an approximation to molecular weight since elution volume depends on molecular shape as well as weight

Table II: Kinetic Data for Yeast HPRT

purine	K_m (M)	V_{max} (mol of IMP L^{-1} s^{-1})
hypoxanthine	1.1×10^{-5}	1.1×10^{-8}
	K_i (M)	
guanine	4×10^{-6}	
6-thioguanine	8×10^{-7}	
adenine	$>1.4 \times 10^{-4}$	
xanthine	$>1.4 \times 10^{-4}$	

and other factors such as sample dilution and viscosity (Ackers, 1964). Thus, the enzyme is a dimer of M_r ~55 000, composed of two subunits, probably identical, but at least of identical molecular weight.

The evidence that the species of M_r 29 500 is indeed HPRT is strong. When purified enzyme was applied to a Sephadex G-100 column, NaDodSO$_4$–polyacrylamide gel electrophoresis of fractions containing HPRT activity showed a single protein species with M_r 29 500; the intensity of Coomassie blue staining was proportional to the enzyme activity in each fraction. Immunological evidence also supports the M_r 29 500 molecular weight structure for denatured HPRT. The antiserum precipitates all HPRT activity from crude yeast extracts; thus the antibody recognizes, quantitatively, all HPRT activity present in the cells. The antibody preparations are specific for native HPRT as evidenced by the single immune precipitation reaction seen by Ochterlony analysis. The antibody also recognizes denatured HPRT as shown by a strong line on Ochterlony analysis as well as antibody binding to denatured HPRT transferred from polyacrylamide gels to nitrocellulose. Thus, by immunological criteria, the M_r 29 500 subunit is antigenically HPRT and not a contaminating species.

The reasons for the differences between our polyacrylamide gel electrophoresis results and those of Schmidt et al. are not apparent. The difference is unlikely to be due to variations between strains of *S. cerevisiae* since the same M_r 29 500 subunit weight was found with yeast from commercial sources and from a laboratory strain. Isoelectric focusing data (Schmidt et al., 1976, 1979) do not support the existence of more than one enzyme in crude yeast extracts. Our immunological studies detect only a single HPRT in yeast. It is unlikely that the M_r 29 500 subunit is the result of proteolytic degradation. Our purification scheme included protease inhibitors throughout the early stages of purification. Furthermore, if proteolytic cleavage were responsible for generating subunits smaller than the monomeric peptide reported by Schmidt et al., the cleavage would have to be highly specific and reproducible to always produce peptides of M_r 29 500 in each of five preparations performed in this laboratory. Of interest, controversy exists concerning the structure of mammalian HPRT as well. Muensch & Yoshida (1977) reported a subunit molecular weight of 45 000 for human HPRT and considered the enzyme a dimer. Other workers have found a subunit approximately half that size. A single subunit of M_r 26 000–27 000 is seen in enzyme purified from human erythrocytes (Holden & Kelley, 1978), mouse liver (Hughes et al., 1975), Chinese hamster brain (Olson & Milman, 1974), and beef brain (Paulus & Bieber, 1980). The native enzymes from these sources have had molecular weight estimates reported in the 75 000–85 000 range, consistent with a trimeric structure. Bifunctional cross-linking reagents have been used with human HPRT, however, and suggest native enzyme may be a tetramer (Holden & Kelley, 1978). Yeast enzyme, therefore, seems analogous to mammalian enzyme in having a subunit of similar molecular weight, but native yeast enzyme is dimeric, not trimeric or tetrameric.

Kinetic studies on yeast HPRT have been reported with both partially purified and extensively purified preparations (see Table II). Miller and Bieber used a 30-fold purified sample to demonstrate that a single enzyme in *S. cerevisiae* catalyzed the formation of GMP and IMP from guanine and hypoxanthine, respectively (Miller & Bieber, 1968). Each purine base competitively inhibited utilization of the other base for nucleotide synthesis. K_m values were in the $(0.7–2) \times 10^{-5}$ M range. Purified HPRT reported here showed similar kinetic behavior with similar Michaelis–Menten parameters. Others have also confirmed these findings with highly purified preparations, with minor differences in K_m values (Schmidt et al., 1979; Ali & Sloan, 1980).

Controversy exists, however, as to the substrate specificity of yeast HPRT. Miller and Bieber reported that neither adenine nor xanthine acted as substrate for the enzyme in *S. cerevisiae*. In contrast, deGroodt et al. studied the azathioxanthine-resistant mutant *purl* in *Schizosaccharomyces pombe* and reported loss of hypoxanthine, guanine, and xanthine, but not adenine, phosphoribosyltransferase (PRT) activities in the mutant yeast extracts (DeGroodt et al., 1971). Furthermore, they found PRT activities for the three bases copurified during a 3-fold purification whereas APRT did not. We have confirmed the lack of xanthine and adenine PRT activity of this enzyme from *S. cerevisiae* in pure preparation. We also report here that 6-thioguanine is a strong competitive inhibitor of yeast HPRT with K_m value nearly 10-fold lower than that of the natural substrate. These features are similar to that seen for mammalian enzyme. One would anticipate that 6-thioguanine would be a potent selective agent in vivo for mutant yeast cells lacking HPRT activity, as has been done with mammalian cells in culture. *S. cerevisiae*, however, is resistant to the drug in concentrations as high as 100-fold above the K_m without any enhancement of toxicity by amphotericin B (data not shown), as has been seen with the pyrimidine analogue 5-fluorocytosine (Medoff et al., 1972).

The antibody against yeast HPRT was raised against both native and denatured forms of the enzyme. In addition to providing information on antigenic differences between the yeast and mammalian enzyme and corroborating the dimeric structure of the enzyme, the antibody also can be used to recognize fragments of the enzyme. Thus, the antibody seems to recognize a number of domains in the molecule, with the molecule in its native or denatured state, and might prove useful for detecting the synthesis of inactive fragments of the enzyme from genes carried in recombinant plasmids containing cloned fragments of the yeast genome. Such antibody methods have already been used to identify bacteria carrying gene sequences for hexokinase, phosphoglycerokinase (Clark et al., 1979), and fatty acid synthetase (Chalmers & Hitzeman, 1980).

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Membrane Topography of the Photosynthetic Reaction Center Polypeptides of *Rhodopseudomonas sphaeroides*†

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ABSTRACT: The topography of the photosynthetic reaction center (RC) polypeptides (H, M, and L) was investigated by proteolysis and radioiodination of membrane vesicles isolated from *Rhodopseudomonas sphaeroides*. Chromatophores, obtained from French-pressed cell lysates, are closed vesicles and oriented inside out with respect to the cytoplasmic membrane (cytoplasmic side out). Spheroplast-derived vesicles (SDVs), obtained after osmotic lysis of lysozyme-treated cells, are oriented right side in (periplasmic side out). α-Chymotrypsin treatment of chromatophores and trypsin treatment of SDVs resulted in cleavage of H. α-Chymotrypsin treatment of SDVs did not cleave H, and trypsin treatment of chromatophores did not consistently cleave this polypeptide. M and L of both vesicles were apparently not affected by these proteases. The SDV trypsin cleavage product of H was identified by α-chymotryptic ^{125}I-labeled peptide mapping and had a molecular weight of 26 000. Membrane surface radioiodination with chloroglycoluril coated on glass tubes resulted in preferential labeling of H and M of SDVs and chromatophores. The radiospecific activities of H, M, and L were higher with labeling of SDVs as compared to labeling of chromatophores. α-Chymotryptic ^{125}I-labeled peptide maps of H, M, and L from surface-radioiodinated SDVs differed from the corresponding maps of these polypeptides from surface-radioiodinated chromatophores. The results indicate the asymmetric exposure of H, M, and L on opposite surfaces of the *R. sphaeroides* membrane. Exposed iodination sites of these polypeptides are more abundant on the periplasmic surface than on the cytoplasmic surface of this membrane.

The photosynthetic membrane system of *Rhodopseudomonas sphaeroides* consists of a network of intracellular vesicles formed as an extension of the cytoplasmic membrane (Lascelles, 1968; Oelze & Drews, 1972). Isolated photosynthetic membrane fractions contain several major polypeptides involved in the harvesting and utilization of light energy. Among these are three polypeptides with estimated molecular weights of 28 000, 24 000, and 21 000, designated H, M, and L, respectively. Together with bacteriochlorophyll *a*, bacteriopheophytin, ubiquinone, and iron, these polypeptides comprise the photochemical reaction center (Feher, 1971; Clayton & Haselkorn, 1972; Okamura et al., 1974). The membranes also contain several polypeptides (at least four) with molecular weights between 8000 and 12 000 (Broglie et al., 1980) which in association with bacteriochlorophyll *a* and carotenoids function in harvesting light.

The topographical arrangement of the reaction center (RC)[1]

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