Tryptic peptide analysis of normal and mutant forms of hypoxanthine phosphoribosyltransferase from HeLa cells

(somatic cell genetics/immunopurification/Aminex A-5 column chromatography)

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ABSTRACT Hypoxanthine phosphoribosyltransferase (HPRT, IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8.) can be purified 5-10,000-fold from extracts of HeLa (human) cells by a three-step procedure consisting of high-speed centrifugation, adsorption to Sepharose-conjugated HPRT antibody, and sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Purified enzyme labeled in vivo with radioactive lysine, arginine, or methionine was digested with trypsin and the tryptic peptides were separated by column chromatography on Bio-Rad cation exchanger Aminex A-5. Less than 50 ng (2 pmol) of HPRT is required to produce a tryptic peptide pattern. A methionine-labeled peptide was identified as the COOH-terminus because it was not labeled with either lysine or arginine. We have compared the tryptic peptide patterns of normal HeLa HPRT and a crossreacting HPRT protein lacking enzyme activity from HeLa mutant H23 [Milman et al. (1976) Proc. Natl. Acad. Sci. USA 73, 4589–4593]. The mutant protein has a new lysine-labeled peptide, but the chromatography patterns of arginine- or methionine-labeled peptides appear identical to those of the normal protein. The appearance of the H23 mutant HPRT protein of a new tryptic peptide provides strong evidence for a mutation in the HPRT structural gene. The tryptic peptide patterns were used to determine the total number of residues of labeled amino acid in the protein, and the values are reasonably consistent with those determined by conventional amino acid analysis of erythrocyte HPRT.

Hypoxanthine phosphoribosyltransferase (HPRT, IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8.) is a purine salvage enzyme that catalyzes the synthesis of IMP or GMP from 5-phosphoribosyl-1-pyrophosphate and hypoxanthine or guanine. Numerous features of the enzyme make it a popular subject of study. Clinically, lack of HPRT activity is responsible for the X-linked Lesch–Nyhan syndrome (1, 2), and altered levels of HPRT are often associated with gout (3). Purine base analogs used in cancer chemotherapy require HPRT to convert them to an active nucleotide form. HPRT is also fundamentally important for the somatic cell geneticist. Methods for the selection of cells lacking or expressing HPRT activity are well documented (ref. 4; see ref. 5 for review), and these selective systems form the basis for killing parental cells in most somatic cell hybridization experiments (6).

Positive selection procedures combined with the fact that the X-linked gene for HPRT apparently mutates as if it were haploid even in polyploid cells make HPRT popular for genetic studies of mutational events in eukaryotes (5). Although Harris (7) suggests that resistance to purine base analogs may occur by nonmutationally induced stable phenotypic changes, HPRT deficiency is correlated in many cases with an alteration in the enzyme protein. Putative somatic cell mutants produce HPRT enzyme with altered temperature sensitivity, altered $K_m$ for a substrate, altered isoelectric pH, or antigenicity without enzyme activity (8–12). Additional proof of a structural gene mutation would be the identification of an altered tryptic peptide in the mutant protein and the determination of the amino acid substitution.

We previously described HeLa mutant H23 (12), which lacks HPRT activity but synthesizes normal amounts of HPRT crossreacting material. The mutant HPRT protein focuses at a more acidic pH on isoelectric focusing gels. In this paper we describe micro methods that enable us to identify an altered tryptic peptide in the mutant H23 HPRT protein.

MATERIALS AND METHODS

Cell Growth Conditions. HeLa cells are grown at 37 °C in an atmosphere of 5% CO2/95% air. Methods for growing cells on plastic petri plates and for the selection of HPRT-deficient mutants or revertants have been previously described (12). When large quantities of cells are desired, they are grown in suspension at a density of 5 to 100 × 106/ml in Joklik’s modified minimal essential medium (Gibco, 13.3 g of powder medium per liter) containing 5% calf serum (Irvine Scientific).

Radioisotope-labeled amino acids are incorporated into HeLa proteins as previously described (12) with minor modifications. A modified formulation of Dulbecco’s modified Eagle’s medium (DME) (13) lacks an amino acid (methionine, lysine, or arginine) and contains 10% calf serum, 22 mM sodium bicarbonate, and 15 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes) buffer adjusted to pH 7.2. The modified DME medium promotes normal growth when the missing amino acid is added. For radioisotope incorporation, the missing amino acid is replaced with radioisotope-labeled amino acid [between 0.2 and 3 mCi of $[^35]S$methionine (2.85 Ci/mmol), $[^3]H$lysine (54 Ci/mmol), or $[^3]H$arginine (7 Ci/mmol) from Schwarz/Mann]. The uptake of radioisotope is generally between 15 and 70%. Cell extracts are prepared as previously described (12).

Purification of HPRT with Sepharose-Conjugated Antibody. The following steps are performed at 0–4 °C. Isotope-labeled crude cell extract is diluted 2- to 5-fold with extraction buffer (10 mM MgCl2, 30 mM KCl, 0.1 mM dithiothreitol, 0.5% Triton X-100, and 10 mM Tris-HCl, pH 7.4) and centrifuged for 2 hr at 96,000 × g in a Spincow type 50 rotor. The high-speed supernatant fraction is removed, and to 2 ml supernatant are added 0.6 ml enzyme buffer (20 mM KCl, 6 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 20 mM Tris-HCl, pH 7.8), 0.4 ml of a solution containing 10% Triton X-100 by volume and 10% sodium deoxycholate by weight, and 1.0 ml of 4 M NaCl. The sample is applied to 0.12 ml of Sepharose-con-
jugated HPRT antibody (12) equilibrated in enzyme buffer containing 1% Triton X-100, 1% sodium deoxycholate, and 1 M NaCl and packed in a column consisting of a 1 ml Glasspak syringe (Becton Dickinson) with a glass fiber plug. The column is washed with 12 ml of the same buffer. The column is then allowed to warm to room temperature (about 23°C) and the remaining steps are performed at room temperature. The column is washed with 3–5 ml of water and the Sepharose-conjugated antibody fraction is eluted with 0.5 ml of 0.2% sodium dodecyl sulfate (NaDodSO4). The eluted sample is lyophilized and re-suspended in 100 μl of sample application buffer containing 10% (vol/vol) glycerol 5% (vol/vol) 2-mercaptoethanol, 0.001% bromophenol blue, and 0.0025 M Tris-HCl, pH 6.8. The sample is applied to an NaDodSO4/polyacrylamide gel in a 5 mm internal diameter glass tube precoated with a 200-fold dilution of Kodak Photoflow, and subjected to electrophoresis utilizing the buffer systems described by Laemmli (14) with minor modifications. A 1.3 cm stacking gel of 4.5% polyacrylamide and a 8.7 cm separating gel of 12.5% polyacrylamide are used. The gels are run at 2 mA per gel until the tracking dye band enters the separating gel, and then at 5 mA per gel until the dye band reaches the bottom of the separating gel. The gel is removed from the tube by gentle application of pressure and sliced into 44–45 slices using a razor blade gel slicer. The gel slices are soaked in 0.5 ml of 0.01% NaDodSO4 for 3 days and the radioactivity of 15–25 μl from each fraction is measured in 10 ml of scintillation fluid (9.1 g of New England Nuclear Omnifluor in 2000 ml of toluene and 1170 ml Triton X-100) in a Packard Tri-Carb with an efficiency of 53% for ³H and 71% for ³⁵S. The isotope-labeled enzyme (NaDodSO4 polyacrylamide gel fraction) is identified as the major peak in the gel (Fig. 1) with a mobility the same as a chymotrypsinogen marker run in a separate gel.

Trypsinization of Isope-Labeled HPRT. The following protocol was performed at 0–4°C to remove NaDodSO4. A sample of isotope-labeled HPRT in 0.01% NaDodSO4 is lyophilized and redissolved in 150 μl of water. Next, 20 μl of a 1 mg/ml solution of bovine serum albumin and 60 μl of 75% trichloroacetic acid are added, and the sample is left for 16–20 hr to allow maximal precipitation of protein. The sample is centrifuged 30 min at 8500 x g in a Sorvall SE-12 rotor, and the supernatant is removed and discarded. The precipitate is washed two times with 200 μl of 1 M HCl and lyophilized to remove traces of HCl.

Trypsin treatment is conducted at room temperature (about 23°C). To the lyophilized sample, 50 μl of 1% ammonium bicarbonate, pH 8.1, and 2 μl of a 1 μg/μl solution of trypsin (Worthington TRTPCK, 217 units/mg) are added, and the mixture is allowed to stand for 4 hr. An additional 50 μl of ammonium bicarbonate buffer and 3 μl of trypsin are added, and the mixture is allowed to stand for an additional 16–20 hr. The trypsinized sample is lyophilized to remove the ammonium bicarbonate, and redissolved in water.

Aminex A-5 Column Chromatography of HPRT Tryptic Peptides. A 2 x 150 mm glass microbore jacketed column (Alexis) is filled with 0.4 ml of Aminex A-5 (Bio-Rad). During column packing and peptide separation, the column is maintained at 50°C. At the end of each peptide separation the column is recycled by washing with successive 5–10 ml volumes of 1 M NaOH; 0.1 M pyridine acetate at pH 3.0; 0.1 M pyridine adjusted with acetic acid to pH 3.0; 3 M pyridine acetate at pH 5.5. The column is then washed extensively (about 15 ml) with 0.1 M pyridine acetate, pH 3.0, until the conductivity reaches 5.5 mho (1 mho = 1 siemens, S). The same column has been used for more than 25 peptide separations.

Before application to the column, a sample of tryptic peptides in water is adjusted to 5% (vol/vol) formic acid with concentrated formic acid. The sample (2–20 μg containing 5–20,000 cpm in 20–200 μl) is applied to the column under 45 pounds/inch² (310 kPa) from a pressurized nitrogen cylinder. All additional fluid is applied by a Milton Roy minipump operating at a flow rate of 6–9 ml/hr and a pressure of 200–400 pounds/inch² (1.4–2.8 MPa). After sample application, approximately 1.7 ml of 0.1 M pyridine acetate (pH 3.0) is passed through the column. Next, a gradient of ionic strength and pH is applied by pumping the effluent from an equal-level two-chamber linear gradient apparatus containing 10 ml of 0.1 M pyridine acetate (pH 3.0) in the mixing chamber and 10 ml of 1.2 M pyridine acetate (pH 5.0) in the other chamber. At the end of the gradient the column is washed with 1.7 ml of 1.2 M pyridine acetate (pH 5.0), 1.7 ml of 2 M Tris-HCl (pH 8.0), and 7.0 ml of 1 M NaOH. The column is then recycled as described above. A conductivity meter (Lab Data Control) with a flow cell of 10 μl is used to monitor the conductivity of the fractions.

Four-drop fractions (0.17 ml) are collected directly into 7 ml plastic mini-scintillation vials placed in test tube racks of an LKB fraction collector. Five milliliters of scintillation fluid and 0.5 ml of water are added to each fraction and the radioactivity is determined by counting for 5 or 10 min in a Packard Tri-Carb liquid scintillation counter with a counting efficiency of 22–26% for ³H and 47–56% for ³⁵S.

RESULTS

HPRT from cell extracts can be purified to 10,000-fold by a simple three-step procedure consisting of high-speed centrifugation, adsorption to Sepharose-conjugated HPRT antibody, and NaDodSO4/polyacrylamide gel electrophoresis. The purification of HPRT is equally successful with radioisotope-labeled extract (Fig. 1) and unlabeled extract (Fig. 2). The following evidence demonstrates that the purified protein on NaDodSO4/polyacrylamide gels is HPRT. The NaDodSO4/polyacrylamide gel fraction migrates as if it has a molecular weight of 25–28,000, which we have previously shown is the subunit molecular weight for HPRT (15). Very little radioactivity or protein occurs in other areas of the gel. When ¹²⁵I-labeled HPRT (16) is mixed with crude HeLa extract, the iodinated enzyme is located with the purified protein peak on the NaDodSO4 gel. Neither the iodinated enzyme nor the cellular HPRT protein peak appears in the NaDodSO4 gel if Sepharose-conjugated preimmune antibody (12) is substituted for the Sepharose-conjugated HPRT antibody. Also, no protein peak is observed for HeLa mutants lacking HPRT crossreacting material.

The amount of radioactivity at each step of a sample purification is listed in Table 1. Approximately 0.01% of the radioisotope-labeled amino acid in a crude extract is recovered in the HPRT NaDodSO4 polyacrylamide gel fraction. In control experiments, 50% of iodinated HPRT added to a crude extract is recovered in this fraction. Therefore, we conclude that HPRT comprises 0.02% of the radioisotope-labeled protein. This value is the same as the weight percentage of HPRT calculated by dividing the specific activity of the enzyme in cell extracts, 4 milliunits/mg, by the specific activity of the purified enzyme, 17.5 units/mg (17).

HPRT is separated from NaDodSO4 by precipitating the protein with 20% trichloroacetic acid in the presence of 100 μg/ml bovine serum albumin. This procedure precipitates 80–90% of the labeled HPRT, and experiments with [³⁵S]NaDodSO4 indicate that at least 98% of the NaDodSO4 is removed. The trichloroacetic acid is extracted with HCl, and the
protein is digested by a total of 5% by weight of trypsin. Approximately 80% of the radioactivity in the HPRT NaDodSO4/polyacrylamide gel fraction is recovered in the lyophilized tryptic peptides.

The chromatography patterns of [3H]lysine-, [3H]arginine-, and [35S]methionine-labeled tryptic peptides of parental wild-type and H23 mutant HPRT are shown in Fig. 3. The amount of radioactivity in each peak and the relative areas of the peaks are presented in Table 2. The patterns are very reproducible and comparisons can be made between separate runs. Reproducibility is important because the cost of incorporation of 14C-labeled amino acids for simultaneous double-label experiments is prohibitive.

We can distinguish 10 major peaks in the chromatography pattern of [3H]lysine-labeled tryptic peptides of wild-type HPRT (Fig. 3A). Peaks 1 and 2 are eluted in the initial application buffer; peaks 3 to 9 are eluted by the pyridine acetate gradient; and a broad peak 10 is eluted by the sodium hydroxide wash. The positions of [3H]lysine-labeled tryptic peptide peaks in the chromatography pattern of the H23 mutant HPRT (Fig. 3B) are identical to that in the wild-type pattern except that a new peak labeled “X” appears in the pattern. In addition to the new peak X, the mutant displays a relative increase in the size of peak 3 and a corresponding decrease in the relative size of peak 10.

The chromatography patterns of wild-type and H23 mutant HPRT [3H]arginine tryptic peptides (Fig. 3C, and D) appear identical. Peak 1 is eluted in the application buffer; a cluster of multiple peaks labeled “2” and a distinct peak 3 are eluted by the pyridine acetate gradient; and a broad peak 4 is eluted by the sodium hydroxide wash. In a separate experiment, free arginine eluted at fraction 101 in the middle of the cluster of arginine peaks.

The chromatography patterns of wild-type and H23 mutant HPRT [35S]methionine tryptic peptides (Fig. 3E and F) also appear identical. Four approximately equal size major peaks are observed, although peak 1 often appears split. Methionine peak 1 occurs at the same location as lysine peak 1; methionine peak 3 occurs at the same location as lysine peak 5; and methionine peak 4 occurs at the same location as lysine peak 7 or one of the cluster of arginine peaks. Methionine peak 2 occurs at a location where there are no lysine or arginine tryptic peptides.
Table 1. Purification of radioisotope-labeled HPRT

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<tr>
<td>1. Crude cell extract</td>
<td>1.6 \times 10^9</td>
<td>1.2 \times 10^9</td>
<td>5.5 \times 10^8</td>
<td>5.2 \times 10^8</td>
<td>3.4 \times 10^8</td>
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<td>2. High-speed supernatant</td>
<td>1.1 \times 10^9</td>
<td>9.2 \times 10^7</td>
<td>3.9 \times 10^8</td>
<td>3.8 \times 10^8</td>
<td>2.6 \times 10^8</td>
<td>1.3 \times 10^8</td>
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<tr>
<td>3. Sepharose-conjugated antibody column eluate</td>
<td>3.6 \times 10^4</td>
<td>2.4 \times 10^4</td>
<td>1.1 \times 10^4</td>
<td>8.5 \times 10^4</td>
<td>8.2 \times 10^4</td>
<td>4.6 \times 10^4</td>
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<tr>
<td>4. NaDodSO_4/polyacrylamide gel eluate</td>
<td>1.9 \times 10^5</td>
<td>1.1 \times 10^5</td>
<td>9.9 \times 10^4</td>
<td>6.4 \times 10^4</td>
<td>1.7 \times 10^4</td>
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HeLa wild-type and H23 mutant cells (about 10^7 cells) were grown in 5 mCi of [^{3}H]lysine, 4 mCi of [^{3}H]arginine, or 1 mCi of [^{35}S]methionine, and radioisotope-labeled HPRT was purified by the three-step procedure described in Materials and Methods. Radioactivities were determined with 2 to 5 \mu l samples and the table presents the total radioactivity in cpm in each fraction.

**DISCUSSION**

We have developed micro methods to analyze the effects of somatic cell mutation in HeLa HPRT, a prototype eukaryotic gene. The procedures enable the immunopurification and tryptic peptide mapping of an enzyme that represents only 0.02% of the cell's soluble protein. Less than 50 ng of radioisotope-labeled protein are required for a tryptic peptide pattern.

The use of radioisotope-labeled amino acids simplifies the

![Aminex A-5 chromatography patterns](image-url)
interpretation of one-dimensional tryptic peptide patterns. Trypsin cleaves on the COOH-terminal side of lysine or arginine residues. Thus, each tryptic peptide should contain only one lysine or one arginine except for the COOH-terminal tryptic peptide which contains neither lysine nor arginine unless the amino acid is the COOH-terminal residue of the protein. By labeling with only one of these amino acids, we can independently observe the chromatography pattern of either the lysine or arginine peptides. Each peptide should contain the same amount of radioactivity, but multiple peptides with similar ionic properties can and do occur at the same location in the elution pattern (Fig. 3), producing peaks of different areas.

The relative areas under the peaks in the lysine or arginine elution profiles indicate the corresponding number of lysine or arginine peptides in the peaks. Integral values should be obtained for the number of peptides in the peak. In general, the data in Table 2 indicate that integral values are obtained when lysine peak 9 or arginine peak 3 are assumed to contain a single labeled amino acid. Nonintegral values in some peaks may be due to modification (for example, deamination) of labeled amino acids (ref. 18; our unpublished observations) which could split the radioactivity for a peptide between two locations. Further studies are required to determine if protein modification is occurring in vitro, during enzyme purification, or during trypsinization, and if alterations in our methods can minimize modification.

When another labeled amino acid (for example, methionine) is incorporated, only those tryptic peptides containing that amino acid appear in the pattern. Sometimes these peaks can be correlated with lysine or arginine peptides. For example, in Fig. 2 methionine peak 3 appears to be identical to lysine peak 5. Potentially, an extension of this type of analysis could be used to determine the number and type of amino acid residues in the tryptic peptides.

Methionine peak 2 occurs at a location where there are no lysine- or arginine-labeled peptides, indicating that this peak is the COOH-terminal tryptic peptide of the protein. This finding is significant because the COOH-terminal peptide should always be altered in chain-termination (amber or ochre) or frameshift mutations. Moreover, it may be possible to identify the COOH-terminal cyanogen bromide cleavage product (cleaved at methionine residues) as a trypsin-resistant fragment.

An interesting feature of the tryptic peptide patterns is that they may be used to determine the total number of residues of the labeled amino acid in the protein. Conventional amino acid analysis of erythrocyte HPRT indicates that the protein contains 12 arginine, 5 to 6 methionine, and 16 lysine residues (17). The data in Table 2 suggest that both wild-type and H23 mutant HeLa HPRT contain 11 arginine and 4 methionine residues. Both the erythrocyte and HeLa wild-type enzymes appear to contain 16 lysine residues, but the H23 mutant HPRT protein may contain an extra lysine residue. Although only one representative experiment is shown in Table 2, consistent results have been obtained in repeated experiments. The appearance in the H23 mutant HPRT protein of a new lysine-labeled peptide provides strong evidence for a mutation in the HPRT structural gene.

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