Identification of the Active Sites of Human and Schistosomal Hypoxanthine−Guanine Phosphoribosyltransferases by GMP-2′,3′-dialdehyde Affinity Labeling†

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ABSTRACT: Labeling of human and schistosomal hypoxanthine−guanine phosphoribosyltransferases (HGPRTases) with GMP-2′,3′-dialdehyde (ox-GMP) results in nearly complete inactivation of the enzymes. Digestion of the [^{3}H]ox-GMP-modified HGPRTases with trypsin followed by high-performance liquid chromatographic fractionation, partial amino acid sequencing, and mass spectral analysis of the labeled peptides revealed that four peptides from each of the two HGPRTases were labeled with ox-GMP. The conclusion from these studies indicates that two segments of the human enzyme protein, Ser 4−Arg 47 and Ser 91−Arg 100, and one region in the schistosomal enzyme, Gly 95−Lys 133, were labeled by ox-GMP. Since the ox-GMP labeling of human HGPRTase was effectively blocked by either GMP or *P*Rib*PP*, whereas that of schistosomal HGPRTase was inhibited only by GMP [Kanaaneh, J., Craig, S. P., III, & Wang, C. C. (1994) *Eur. J. Biochem. 223*, 595−601], the two labeled peptides in human enzyme may be involved in binding to both GMP and *P*Rib*PP* while the one peptide in schistosomal enzyme may be implicated only in GMP binding. We have also confirmed a previous observation [Keough, D. T., Emmerson, B. T., & de Jersey, J. (1991) *Biochim. Biophys. Acta 1096*, 95−100] that carboxymethylation of Cys 22 in the human HGPRTase by iodoacetate was inhibited by *P*Rib*PP*. We also demonstrated that the carboxymethylation of Cys 25 in schistosomal HGPRTase by iodoacetate was specifically blocked by *P*Rib*PP*. Apparently, the N-terminal regions in both enzymes are involved in *P*Rib*PP* binding. The fact that ox-GMP labels the N-terminal region in human enzyme but not in schistosomal enzyme and that *P*Rib*PP* protects against ox-GMP labeling in human enzyme but not in schistosomal enzyme both suggest that the amino-terminal *P*Rib*PP*-binding site may be in close proximity to the GMP-binding site in human HGPRTase but not in schistosomal HGPRTase. This clear distinction between the active sites of human and schistosomal HGPRTases could be further exploited for potential opportunities for antischistosomal chemotherapy.

Hypoxanthine−guanine phosphoribosyltransferase (HGPRTase;[1] EC 2.4.2.8) is a purine salvage enzyme which catalyzes the transfer of a phosphoribosyl group from 5-phosphoribosyl-1-pyrophosphate (*P*Rib*PP*) to the N9 nitrogen of purine bases hypoxanthine or guanine to form their respective mononucleotides (IMP or GMP) and pyrophosphate (PP_i). HGPRTases of human and the parasitic trematode, *Schistosoma mansoni*, are both of biomedical interest. In humans, severe deficiency of HGPRTase activity results in the Lesch−Nyhan syndrome (Seegmiller et al., 1967) whereas partial deficiency causes hyperuricemia which may lead to gouty arthritis and nephrolithiasis (Kelley et al., 1967). Due to their inability to synthesize purines *de novo*, schistosomal parasites rely on the salvage of exogenous purine bases into the corresponding purine nucleotides needed for cellular metabolism. The schistosomal HGPRTase is a crucial enzyme for the survival of this pathogen and therefore has been proposed as a potential target for antischistosomal chemotherapy (Senft & Crabtree, 1983; Dovey et al., 1984).

Complementary DNAs encoding the human HGPRTase (Free et al., 1990; Davidson et al., 1993) and schistosomal HGPRTase (Craig et al., 1988) have been expressed in *Escherichia coli* at high levels as soluble, enzymatically active recombinant enzymes (Craig et al., 1991; Kanaaneh et al., 1994). A comparison of the schistosomal and human HGPRTases is essential in the design of inhibitors that specifically target the parasite enzyme. Comparative studies on both recombinant enzymes have shown that the schistosomal HGPRTase differs significantly from the human enzyme in the kinetics of their catalyzed reactions (Yuan et al., 1992) and thermostability (Yuan et al., 1993). In addition, using the affinity label GMP-2′,3′-dialdehyde (ox-GMP), we have shown that the two enzymes differ in the ways of being labeled by ox-GMP. While the ox-GMP labeling of human HGPRTase can be effectively protected by *P*Rib*PP*, the labeling of schistosomal enzyme cannot be blocked by *P*Rib*PP* at all (Kanaaneh et al., 1994).

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[1] Abbreviations: HGPRTase, hypoxanthine−guanine phosphoribosyltransferase; OPRTase, orotate phosphoribosyltransferase; *P*Rib*PP*, 5-phosphoribosyl-1-pyrophosphate; ox-GMP, GMP-2′,3′-dialdehyde; DTT, dithiothreitol; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; CMC, *S*-(carboxymethyl)cysteine; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption ionization; HPLC, high-performance liquid chromatography.

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In human HGPRTase, the amino-terminal region, corresponding to the first 120 amino acids, has been proposed to contain the *P*Rib*PP* binding site by comparison with the primary structures of other two PRTases: *Salmonella typhimurium* ATP PRTase and *E. coli* glutamine PRTase (Argos et al., 1983). In agreement with this hypothesis, Keough et al. (1991) have shown that human HGPRTase is specifically protected by *P*Rih*PP* against iodoacetate labeling of Cys 22 among its four cysteine residues. In a closely related enzyme, the *S. typhimurium* orotate phosphoribosyltransferase (OPRTase), *P*Rib*PP* was found also protecting Lys 26 against chemical modifications (Grubmeyer et al., 1993). Thus, the N-terminal portions of the two enzymes are likely involved in forming the *P*Rib*PP* binding sites.

Recently, the three-dimensional structures of recombinant human HGPRTase with bound GMP (Eads et al., 1994) and of *S. typhimurium* OPRTase complexed with OMP (Scapin et al., 1994) have each been solved and refined to 2.5 Å resolution using X-ray diffraction data. In addition, the structure of a third member of the PRTases family, glutamine–amido phosphoribosyltransferase (amido-PRTase; Smith et al., 1994), was also solved using multiwavelength anomalous diffraction (MAD). In the crystal structures of the three PRTases, a similar core structure, which resembles a dinucleotide-binding fold, has been observed. Sequences corresponding to the core structure are located in the middle of the primary structure of HGPRTase, OPRTase, and the PRTase domain of amido-PRTase. In the HGPRTase and OPRTase crystal structures, the role of the putative *P*Rib*PP*-binding motif, identified previously in amino acid sequence comparisons as residues Val 129–Lys 140 in HGPRTase and Val 126–Thr 131 in OPRTase (Hove-Jensen et al., 1986; Wilson et al., 1983), was found to bind the 5′-phosphate group of GMP or OMP. In human HGPRTase, the ribose O3′ of GMP is within hydrogen bond distance from the carboxylate group of Glu 133 and the O2′ is 3.6 Å from the amino nitrogen of the side chain of Lys 68. However, in the OPRTase crystal structure, the O2′ and O3′ hydroxyl groups of the bound OMP form hydrogen bonds with the ϵ-amino group of Lys 26. Thus, the crystal structure of OPRTase agrees with the biochemical data that Lys 26 is involved in the active site, whereas the crystal structure of human HGPRTase is in apparent disagreement with the previous postulation that Cys 22 should be involved in *P*Rib*PP* binding.

In our present studies, ox-GMP and iodoacetate were used in an attempt to label the *P*Rib*PP*- and GMP-binding sites in both human and schistosomal HGPRTases. In the human HGPRTase, ox-GMP labeled two regions, Ser 4–Arg 47 and Ser 91–Arg 100. This labeling was inhibited by either GMP or *P*Rib*PP*. We also confirmed the observation by Keough et al. (1991) that carboxymethylation of Cys 22 by iodoacetate was inhibited by *P*Rib*PP*. In the schistosomal HGPRTase, ox-GMP labeled only one stretch from Gly 95 to Lys 133, away from the amino-terminal portion of the protein. These labelings were inhibited by GMP but not by *P*Rib*PP*, while the latter was shown to protect the Cys 25 in schistosomal enzyme against iodoacetate labeling. Together, the biochemical data have provided a self-consistent suggestion that the amino-terminal portions of both human and schistosomal HGPRTases are involved in *P*Rib*PP* binding. The active pocket in human HGPRTase is apparently more compact than that in the schistosomal enzyme, which may explain why ox-GMP labels the amino-terminal portion and why *P*Rib*PP* competes with it in the human HGPRTase whereas both fail to happen in the schistosomal enzyme. That ox-GMP cannot label the amino-terminal portion of schistosomal HGPRTase is also consistent with the previous finding that ox-GMP labeling of the schistosomal enzyme could not be blocked by *P*Rib*PP* (Kanaaneh et al., 1994).

MATERIALS AND METHODS

Materials. GMP, GMP-2′,3′-dialdehyde (ox-GMP), 5-phosphoribosyl-1-diphosphate (*P*Rib*PP*), sodium borohydride, sodium periodate, iodoacetic acid, and trifluoroacetic acid (TFA) were purchased from Sigma in the highest purities available. L-1-(Tosylamido)-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin was from Worthington Biochemical Corp., Freehold, NJ. Acetonitrile used in reverse-phase HPLC was purchased from Fisher. [8-^{3}H]GMP (8.9–14.2 Ci/mmol) was obtained from Moravek Biochemicals. Iodo[2-^{14}C]acetic acid (50 mCi/mmol) and iodo[2-^{3}H]acetic acid (150 mCi/mmol) were from Amersham International. All other solvents and chemicals were of analytical grade.

General Methods. The recombinant schistosomal HGPRTase was purified from low-phosphate-induced *E. coli* strain SØ606 (Δ*gpt-pro-lac, thi, hpt*) transformed with the pBSprt expression plasmid (Craig et al., 1991) as described previously (Yuan et al., 1992). The recombinant human HGPRTase was purified from the same strain of *E. coli* transformed with pBAcprt expression plasmid by a previously described procedure (Kanaaneh et al., 1994). Protein concentrations were determined by the Bio-Rad Bradford protein assay using bovine serum albumin as a standard. Molar concentrations of the pure HGPRTases were calculated using a molecular mass of 24 kDa for the human enzyme monomer and 26 kDa for the schistosomal enzyme monomer as determined by mass spectrometry. The HGPRTase activity was assayed as described previously (Yuan et al., 1990).

Labeling of HGPRTase with [8-^{3}H]GMP-2′,3′-dialdehyde. [8-^{3}H]ox-GMP was synthesized from [8-^{3}H]GMP (70–112 μM in 50% ethanol) by oxidation with a 20-fold molar excess of sodium periodate dissolved in 100 mM sodium phosphate (pH 7.0) in a total volume of 600 μL. The reaction was allowed to proceed for 1 h at room temperature in the dark and terminated by adding a 2-fold molar excess of ethylene glycol (3 mM) over periodate during 20 min at room temperature. For monitoring the time course and stoichiometry of HGPRTase inactivation with [8-^{3}H]ox-GMP, human HGPRTase (27.9 nmol of the monomer) was incubated at 4 °C with 741.1 nmol of [8-^{3}H]ox-GMP (0.4 Ci/mmol) in 1580 μL of 50 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$, and 1 mM dithiothreitol (DTT). Schistosomal HGPRTase (19.2 nmol of the monomer) was incubated at 4 °C with 568.2 nmol of [8-^{3}H]ox-GMP (0.7 Ci/mmol) in 1860 μL of the same buffer. Aliquots of 150 μL were withdrawn at different times, reduced with 5 mM sodium borohydride for 10 min, and loaded on a Quick Spin Protein column (Boehringer Mannheim) preequilibrated with 4 mL of the 50 mM Tris-HCl buffer. An additional 150 μL of the Tris-HCl buffer was applied to the resin bed after the protein sample had been absorbed, and the column was centrifuged at 900*g* for 3 min. The resulting eluate fractions (400 μL)

were assayed for enzymatic activity, protein concentration, and radioactivity. A control was run in parallel with the [8-^{3}H]ox-GMP omitted. Using this technique 96−97% of the free [8-^{3}H]ox-GMP was retained on the column. To label the HGPRTases for tryptic digestion, the reaction mixture containing 22−34 nmol of [8-^{3}H]ox-GMP (14.2 Ci/mmol) was incubated with 20 nmol of purified HGPRTase in 1 mL of the buffer mentioned above. After 15 h of incubation at 4 °C, the reaction mixture was treated with 10 mM $NaBH_4$ at 0 °C for 1 h. The modified protein was separated from the unbound radiolabeled ox-GMP and the excess $NaBH_4$ by gel filtration using a prepacked PD-10 column of Sephadex G-25 (Pharmacia) equilibrated with approximately 25 mL of 10 mM NH_4HCO_3 (pH 7.8). Protection experiments were performed by preincubating the enzyme with 1 mM GMP or *P*Rib*PP* for 30 min at 4 °C before adding [8-^{3}H]ox-GMP.

Labeling of HGPRTase with Iodoacetate. Labeling of human HGPRTase (300 μg, 12.5 nmol) with [^{14}C]iodoacetate (7746 dpm/nmol; 7.1 mM final concentration) was carried out at 25 °C in 1 mL of 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 1 mM β-mercaptoethanol, following the procedure of Keough et al. (1991). The incubation was allowed to proceed for 50 h. Schistosomal HGPRTase (300 μg, 11.5 nmol) was reacted with [^{3}H]iodoacetate (30986 dpm/nmol; 7.1 mM final concentration) under the same conditions. Excess iodoacetate was removed from both samples by repeated dialysis against 20 mM NH_4HCO_3 (pH 7.8) at 5 °C using Spectra/Por 2 membrane. Dialysis was continued until the dialysate showed less than 2-fold of the background radioactivity.

Tryptic Digestion of Human and Schistosomal HGPRTases. The volume of the protein solution after desalting by gel filtration or dialysis was reduced by lyophilization to give a protein concentration of 1 mg/mL in 80 mM NH_4HCO_3 (pH 7.8). After 30 min of incubation at 37 °C with 8 M urea, the volume of this solution was adjusted with 80 mM NH_4HCO_3 (pH 7.8) to contain 2 M urea. The HGPRTases were incubated with TPCK-treated trypsin at a molar ratio of 20:1. Digestion was allowed to proceed at room temperature. A second dose of trypsin was added after 12 h, and the reaction was allowed to continue for another 5 h.

Peptide Separation and Sequencing. The peptides resulting from 250 μg of the trypsin-digested protein were separated by reverse-phase HPLC on a Rainin Microsorb-MV C-18 300 Å column (5 μm, 4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid (buffer A). Peptide fractionations were accomplished with a linear gradient from 0% to 75% acetonitrile in 0.08% trifluoroacetic acid (buffer B) at a flow rate of 1 mL/min using a Rainin HPXL gradient HPLC system and a Dynamax UV-1 absorbance detector operating at 214 nm. Radiolabeled peptides were located by counting aliquots from 0.5−1 mL fractions with a Beckman LS-3801 scintillation counter. Amino acid sequence determination was performed by Edman degradations at the Biomolecular Resource Center at UCSF using an Applied Biosystems 470A gas-phase sequencer. The phenylthiohydantion (PTH) derivatives were identified and quantitated by reverse-phase HPLC using an on-line Applied Biosystems 120A PTH analyzer.

Mass Spectrometry. Molecular masses of all tryptic peptides were determined by electrospray ionization (ESI) mass spectroscopy on a VG Quattro BQ mass spectrometer. Molecular masses of intact human and schistosomal HGPRTases were determined by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. For human HGPRTase, the analysis was performed on a VG TofSpec (Fisons Instruments) MALDI mass spectrometer equipped with a nitrogen laser and operated in the linear mode. For the schistosomal enzyme, the analysis was performed on a Kratos KOMPACT MALDI III LD/TOF mass spectrometer (Kratos Analytical) operating at 20 kV accelerating potential in the positive ion linear mode. All MALDI spectra were externally calibrated.

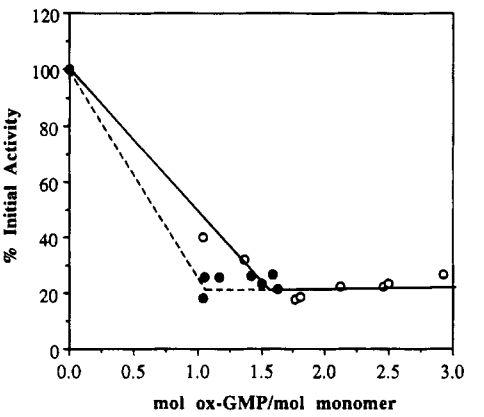

FIGURE 1: Stoichiometry of ox-GMP labeling and inactivation of HGPRTases. Time samples: human HGPRTase (○), 10, 25, 40, 70, 130, 190, 250, and 370 min; schistosomal HGPRTase (●), 10, 25, 40, 55, 70, 115, 175, and 295 min.

RESULTS

Affinity Labeling of Human and Schistosomal HGPRTases with GMP-2′,3′-dialdehyde. We have previously shown that ox-GMP (50 μM) inactivates irreversibly and specifically both the human and schistosomal HGPRTases (0.35 μM) in a time-dependent manner, reaching about 85% inactivation within 4 h at 0 °C (Kanaaneh et al., 1994). To determine the time course and stoichiometry of labeling by [8-^{3}H]ox-GMP, human and schistosomal HGPRTases were incubated at 4 °C with a 26.6-fold and a 29.6-fold molar excess of [8-^{3}H]ox-GMP, respectively. Time samples of the labeled enzyme were taken for analyses of enzyme activities and enzyme-bound [8-^{3}H]ox-GMP and protein concentrations. The results (not shown) indicated that within 10 min of incubation, which was the shortest time limit experimentally possible, 60% of the human HGPRTase and 80% of the schistosomal HGPRTase activities were lost. Approximately one molecule of [8-^{3}H]ox-GMP was bound to each monomer of either enzyme at this time point (see Figure 1). Further incubation of the human enzyme up to 40−70 min resulted in 80% loss of the enzyme activity and 1.5 molecules of [8-^{3}H]ox-GMP bound per monomer. Prolonged incubation beyond this point saw little additional inactivation, but the molar ratio between bound [8-^{3}H]ox-GMP and the enzyme monomer reached 3 at 370 min. Prolonged incubation with the schistosomal enzyme did not result in further inactivation, but the [8-^{3}H]ox-GMP/monomer ratio reached 1.5 after 295 min (Figure 1). In a similar experiment, the HGPRTases incubated with a 30-fold molar excess of nonradioactive ox-GMP for 15 h were analyzed in laser energy mass spectrometry. The results in Figure 2A show that 3 mol of ox-GMP is bound to 1 mol of human HGPRTase monomer.

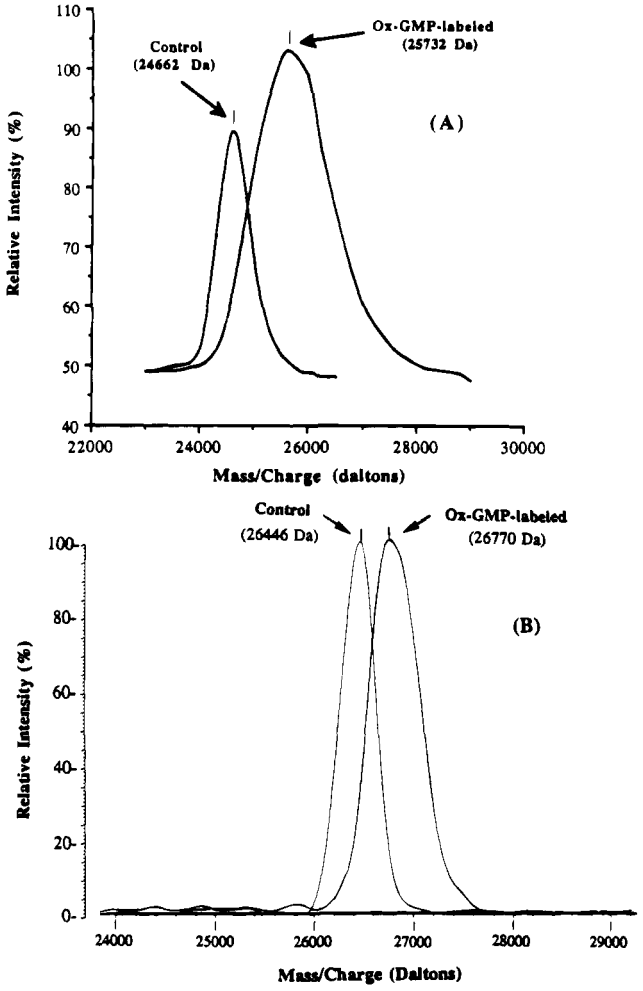

FIGURE 2: Stoichiometry of ox-GMP labeling of human (panel A) and schistosomal (panel B) HGPRTases. Human HGPRTase (33 nmol of the monomer) was incubated for 15 h at 4 °C in 1 mL of 50 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, and 1 mM DTT, in the presence or absence of 1 mmol of ox-GMP. Schistosomal HGPRTase (15.4 nmol of the monomer) was incubated under the same conditions in the presence or absence of 500 nmol of ox-GMP. The reaction mixture was then treated with 10 mM $NaBH_4$ for 1 h at 0 °C. Excess $NaBH_4$ and unbound ox-GMP were removed using Sephadex G-25 prepacked columns equilibrated with 25 mL of double distilled water. Three microliter aliquots were taken and assayed for enzyme activity as described previously (Yuan et al., 1990). Analysis by MALDI mass spectrometry was performed as described in Materials and Methods. The limits of resolution by MALDI mass spectrometry in the 24−27 kDa range is 200 (full width at half-height). The results shown represent one of four different determinations taken for each of the control and the labeled enzyme.

The width of the ox-GMP-labeled peak is 2−3 times that of the control, indicating heterogeniety among the labeled protein molecules. By the same mass spectral analysis, only 1 mol of ox-GMP is bound to 1 mol of schistosomal HGPRTase monomer with about 25% broadenings of the peak (Figure 2B).

Isolation and Partial Sequencing of [^{3}H]GMP-2′,3′-dialdehyde-Labeled Peptides. To determine the region of the HGPRTase covalently modified by [^{3}H]ox-GMP, human and schistosomal HGPRTases were labeled overnight at 4 °C with only a 2-fold molar excess of [^{3}H]ox-GMP. Under these conditions, the enzyme activities were inhibited by about 80%, and the amount of [^{3}H]ox-GMP label incorporated was lowered to 1.3 mol/mol of human HGPRTase

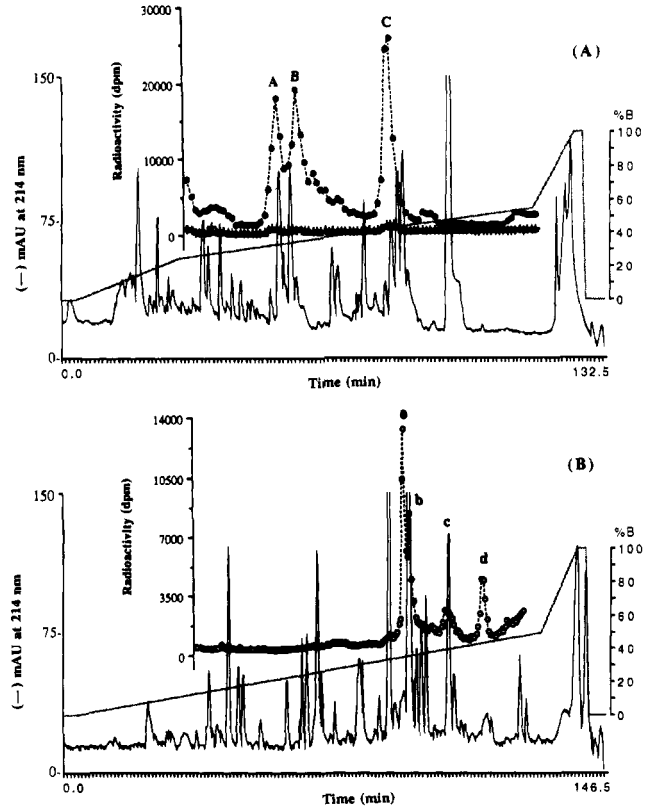

FIGURE 3: Reverse-phase HPLC separation of tryptic digests of [^{3}H]ox-GMP-labeled human (panel A) and schistosomal (panel B) HGPRTases. Both enzymes were labeled, desalted by gel filtration, denatured with 8 M urea, digested with trypsin, and subjected to HPLC. The peptides resulting from digestion of 250 μg of human HGPRTase (panel A) were eluted at 1 mL/min with a linear gradient from 25% to 55% solvent B over 85 min. The peptides resulting from digestion of 250 μg of schistosomal HGPRTase (panel B) were eluted at 1 mL/min with a linear gradient from 0% to 50% solvent B over 128 min. Protection experiments (panel A) were performed by preincubating the human enzyme in the absence (●) or presence of 1 mM GMP (▲) or *P*Rib*PP* (+) for 30 min at 4 °C before adding [^{3}H]ox-GMP. The tritium label was followed by scintillation counting of 10 μL aliquots from 1 mL (panel A) or 0.5 mL (panel B, ○) fractions. mAU, milliabsorbance units.

monomer but maintained at 1 mol/mol of schistosomal HGPRTase monomer. After proteolysis of the labeled enzyme with trypsin, peptides were separated in HPLC. Five preparative runs were repeated on each enzyme sample, and the presence of three radioactive peaks, A−C, (Figure 3A) from the human enzyme and four peaks, a−d (Figure 3B), from the schistosomal enzyme was reproducibly demonstrated. For each enzyme, the combined radioactivity from these peptide peaks represented about only 15% of that originally applied to the HPLC column, suggesting instability of the label under the conditions of HPLC. The radiolabel was subsequently stabilized in the collected effluent by neutralizing the pH from 2.0 to 7.8 with 50 mM ammonium bicarbonate (pH 7.8). The fractions with the highest radioactivity from each of these peaks were combined, concentrated by lyophilization, and repurified on the same C-18 reverse-phase column.

For human HGPRTase, peptides associated with peaks A and C (Figure 3A) were identified by N-terminal amino acid sequencing of the first five amino acids. Peak A contains a peptide of VFIPH... with an initial yield of 50 pmol. Peak C contains a peptide of SPGVV... with an initial yield of 47 pmol. Peak A and peak C contain 39.6 and 41.0 pmol of

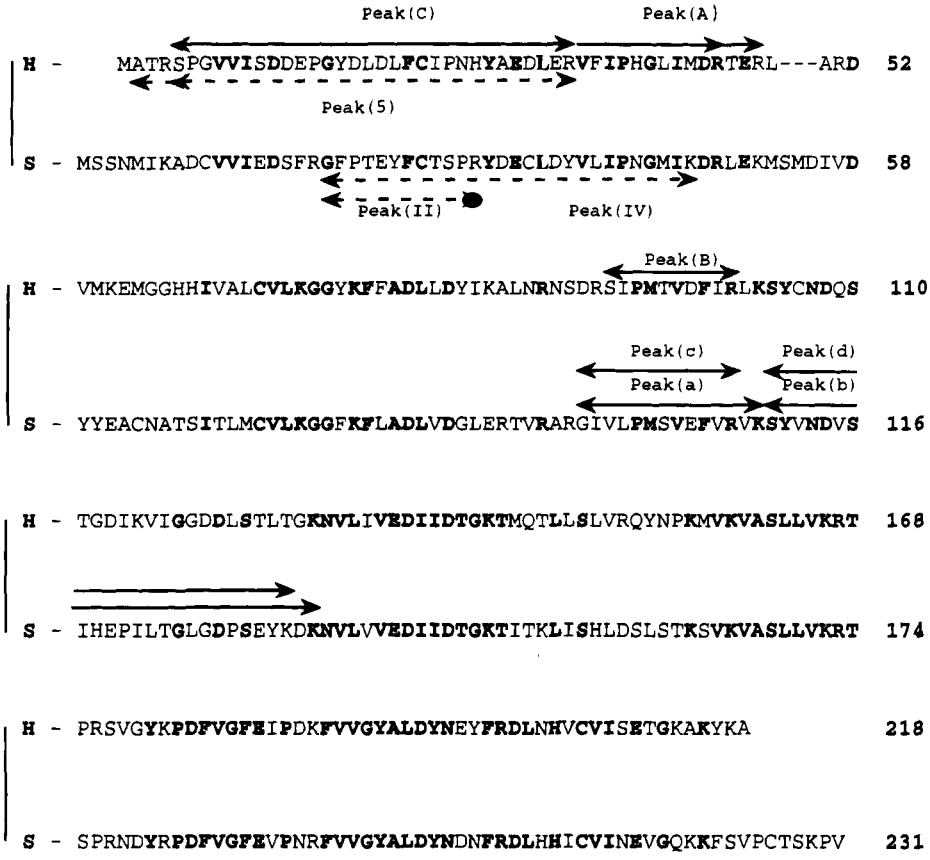

FIGURE 4: Alignment of amino acid sequences for the HGPRTases of humans (H) [see Jolly et al. (1983)] and schistosomes (S) [see Craig et al. (1988)]. Bold letters indicate identical amino acids. Peptides labeled with [^{3}H]ox-GMP are indicated by solid lines drawn above the sequence. Those labeled with iodoacetate are indicated by broken lines drawn below the sequence.

ox-GMP, respectively. Thus, the molar ratio of ox-GMP to peptide is 0.79 for peak A and 0.87 for peak C. In order to determine the sizes of these peptides, each sample was subjected to mass spectroscopic analysis, using electrospray ionization (ESI) mass spectroscopy. Peak A was shown to contain two tryptic peptides, while peak C was found to contain a single tryptic peptide (Table 1). The major peptide in peak A represents amino acids Val 34−Arg 44, and the minor peptide represents its extended form Val 34−Arg 47 (Figure 4). The peptide in peak C represents Ser 4−Arg 33. Further purification of peak A by HPLC with a shallower gradient resolved the two radioactive peptides (Figure 5A), while peak C yielded still only one radioactive peptide (Figure 5B). Peak B was concentrated by lyophilization and subjected to sequence and mass spectral analysis without further purification due to its relatively low quantity. Partial sequencing showed that peak B contained a peptide of SIPMT... with an initial yield of 26 pmol containing 21.6 pmol of ox-GMP, which corresponds to a ratio of ox-GMP to peptide of 0.83. Mass spectral analysis (Table 1) showed that it represents the peptide Ser 91−Arg 100. For all the three labeled peptides, the results from the mass spectral analysis agree totally with the partial amino acid sequencing data. The ox-GMP label, though capable of partially surviving HPLC and sustaining the laser desorption ionization in mass spectroscopy (Figure 2), is, however, labile in the ESI mass spectroscopy. The ESI mass spectrometry was operated in the positive ion mode; we were unable to detect the decomposed negatively charged adduct (ox-GMP). Thus, the peptides identified in ESI mass spectrometry were each without the ox-GMP label. However, the close-to-unity ratios between the peptide and ox-GMP and the purity of each labeled peptide sample from HPLC suggest that peptides A, B, and C were each covalently linked to ox-GMP in their original state. This linkage is probably formed between the aldehyde groups of ox-GMP and the guanidino group of the C-terminal arginine residue in each of the peptides. Ox-GMP labeling of arginine residues via Schiff base formation was reported to occur in previous cases (King, 1966; Signor et al., 1971; Hountondii et al., 1990). Trypsin apparently still recognizes the ox-GMP-modified arginine residues and digests the peptide bond accordingly (Signor et al., 1971). We have previously shown that inactivation and labeling of human HGPRTase with ox-GMP were inhibited by pre-incubation with GMP or *P*Rib*PP* (Kanaaneh et al., 1994). To determine whether any of the identified peptides labeled by [^{3}H]ox-GMP can be protected by GMP or *P*Rib*PP*, we repeated the same experiments in the presence of 1 mM GMP or *P*Rib*PP*. The plots in Figure 3A show that both GMP and *P*Rib*PP* provided excellent protection for all the three peptides, A−C, against ox-GMP labeling. Thus, the ox-GMP-labeled peptides in the human HGPRTase are (A) Val 34−Arg 44 and Val 34−Arg 47, (B) Ser 91−Arg 100, and (C) Ser 4−Arg 33 (see Figure 4). There are three sites labeled by ox-GMP, even though the molar ratio between the label and the enzyme is only 1.3. This suggests that ox-GMP labels any one of the three sites at random, but a single site labeling can lead to enzyme inactivation. The data in Figure 1 suggest also that once one of the three sites becomes labeled by ox-GMP, the labeling of the other two sites proceeds at much slower rates, hinting steric hindrance caused by the single ox-GMP molecule.

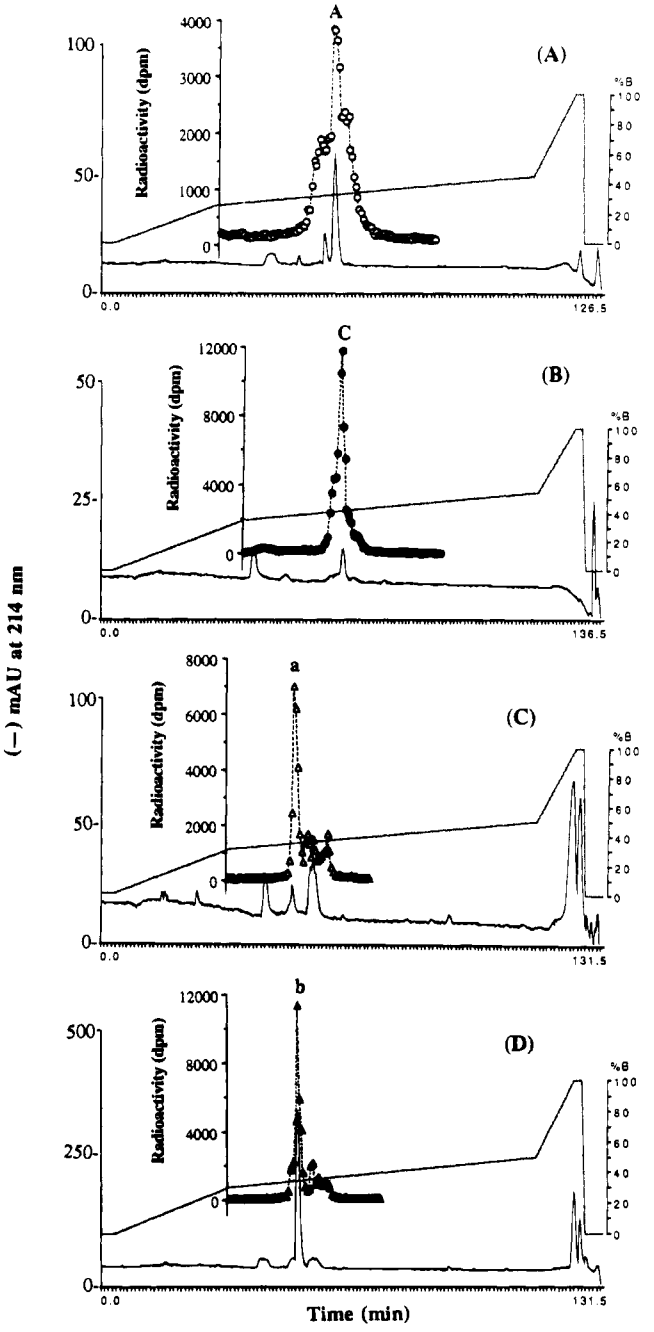

FIGURE 5: Rechromatography of tryptic peptides from radioactive peaks A and C (panels A and B) and from radioactive peaks a and b (panels C and D) from the initial separation of the tryptic digest of [^{3}H]ox-GMP-labeled human and schistosomal HGPRTases, respectively. Radioactive fractions from peak A or C from three injections as shown in Figure 3A were each pooled, concentrated by lyophilization, and injected onto the C-18 reverse-phase column. Peak A was eluted at 1 mL/min with a linear gradient of 25−45% solvent B over 80 min. Peak C was eluted with a linear gradient of 35−55% solvent B over 80 min. The fractions containing peaks a and b each obtained from five injections as shown in Figure 3B were pooled, concentrated by lyophilization, and injected onto the C-18 reverse-phase column. Both peaks a and b were eluted at 1 mL/min with a linear gradient from 30% to 50% solvent B over 80 min. The tritium label was monitored by counting 10 μL aliquots out of 0.5 mL fractions. mAU, milliabsorbance units.

For the [^{3}H]ox-GMP-labeled schistosomal HGPRTase peptides a−d (Figure 3B), further purifications resulted in a single radioactive peak in each of the four peptide samples (Figure 5 and data not shown). The peptides in each of the four peaks were identified by N-terminal amino acid sequencing of the first five amino acids in each peptide. Peak a contained a peptide of GIVLP... with an initial yield of 69 pmol containing 55.8 pmol of ox-GMP. Thus, the molar ratio of ox-GMP to peptide is 0.81. Peak b contained two different peptides. Data from amino acid sequencing and mass spectral analysis suggest that they are Phe 192−Arg 205 and Ser 109−Lys 133 (Table 1 and data not shown). Further purification of peak b by HPLC using a linear gradient of acetonitrile in 10 mM ammonium acetate (pH 5.8) yielded a single species of radiolabeled peptide that was identified to be Ser 109−Lys 133. The initial yield of this peptide was 41 pmol containing 11 pmol of ox-GMP, which corresponds to a ratio of ox-GMP to peptide of 0.27. Peptide c was identified by partial amino acid sequencing and mass spectral analysis to contain the peptide Gly 95−Arg 106 (Table 1). The stoichiometry of labeling by ox-GMP for this peptide was only 0.01. Peak d contained a peptide of SYVND... with an initial yield of 12 pmol containing 11 pmol of ox-GMP, which corresponds to a ratio of ox-GMP to peptide of 0.92. Partial amino acid sequence analysis showed that the radioactive peptides in peaks b and d share the same N-terminal five amino acid residues, which correspond to Ser 109−Asp 113. A similar observation was made on the radiolabeled peptides in peaks a and c, which share the initial sequence Gly 95−Pro 99. The slower emergence of peptides c and d from HPLC suggests that they are the shorter forms of a and b, respectively, and may correspond to the amino acid residues Gly 95−Arg 106 and Ser 109−Lys 131 (see Figure 4). Thus, in conclusion, the ox-GMP-labeled peptides in schistosomal HGPRTase are (a) Gly 95−Lys 108, (b) Ser 109−Lys 133, (c) Gly 95−Arg 106, and (d) Ser 109−Lys 131 (see Figure 4). Due to the overlaps between peptides a and c and peptides b and d, there are most likely two sites in schistosomal HGPRTase subjecting to ox-GMP labeling. Again, since the molar ratio between the label and the enzyme is one, ox-GMP can apparently label any one of the two sites at random and result in enzyme inhibition. The labeling of schistosomal HG-

Table 1: Electrospray Ionization (ESI) Mass Spectrum of [^{3}H]ox-GMP-Labeled Peptides from Human and Schistosomal HGPRTases

	mass/charge	charges	molecular mass	fragment
human HGPRTase				
peak A				
component A	433	3	1297	
	650	2	1298	Val 34−Arg 44
component B	422	4	1684	
	562	3	1684	
	843	2	1684	Val 34−Arg 47
peak B				
	590	2	1179	
	1180	1	1179	Ser 91−Arg 100
peak C				
	1691	2	3379	
	1128	3	3380	
	845	4	3378	Ser 4−Arg 33
schistosomal HGPRTase				
peak b				
component A	565	3	1693	
	847	2	1693	Phe 192−Arg 205
component B	695	4	2778	
	927	3	2777	Ser 109−Lys 133
peak c				
	674	2	1347	
	1347	1	1346	Gly 95−Arg 106

PRTase monomer by a second ox-GMP proceeds at an extremely slow rate (Figure 1), suggesting steric hindrance caused by the first ox-GMP molecule.

Identification of the Iodoacetate-Modified Cysteines in HGPRTases That Are Protected by PRibPP. In order to identify the cysteine residues in both human and schistosomal HGPRTases that may be protected by *P*Rib*PP* against carboxymethylation by iodoacetate, each enzyme (11.5−12.5 μM) was incubated with 20 mM *P*Rib*PP* plus 10 mM $MgCl_2$ and then reacted with 7.1 mM radiolabeled iodoacetate for 50 h at 25 °C. Each monomer of the human HGPRTase, which contains four cysteine residues, was labeled with 1 molecule of iodoacetate when *P*Rib*PP*−Mg^{2+} was present but with 1.4 molecules of iodoacetate in the absence of *P*Rib*PP*−Mg^{2+} under the same experimental conditions. For each monomer of the schistosomal HGPRTase, which contains 7 cysteine residues, only 0.5 molecule of iodoacetate was associated with each monomer in the presence of *P*Rib*PP*−Mg^{2+}, whereas in the absence of *P*Rib*PP*−Mg^{2+} it contained 1 molecule of iodoacetate. Trypsin digestion of the radiolabeled protein showed in reverse-phase HPLC the profile of radioactivity represented by six radioactive peaks from human HGPRTase without *P*Rib*PP*−Mg^{2+}. Only one (peak 5) of the six peaks lost the radiolabel in the presence of *P*Rib*PP*−Mg^{2+} (Figure 6A). This peak was a mixture of two peptides: Ala 1−Arg 33 at a level of 140−150 pmol and Ser 4−Arg 33 at a level of 25−30 pmol. On the basis of the known sequence of the human HGPRTase, both peptides contain the same cysteine residue, Cys 22 (Figure 4).

Figure 6B shows the HPLC profiles of the tryptic digest of iodoacetate-labeled schistosomal HGPRTase. There are four major radioactive peaks in the absence of *P*Rib*PP*−Mg^{2+}, two of which (peaks II and IV) have their radioactivities reduced by more than 90% by the presence of *P*Rib*PP*−Mg^{2+}. The site of modification in the radioactive peptides was identified by Edman degradation in which the radioactivity recovered from the first nine cycles was counted (data not shown). There is a single peptide in peak II, and there are two peptides in peak IV. The peptide in peak II has the sequence GFPTGTF(CMC)T..., where CMC stands for *S*-(carboxymethyl)cysteine. During the Edman degradations, radioactivity started to appear at cycle 8 and continued to rise at cycle 9 in a manner consistent with Edman degradation carryover resulting from incomplete amino acid cleavage in cycle 8. The product obtained from cycle 8 was identified as PTH−(*S*-carboxymethyl)cysteine (CMC) by HPLC (data not shown). Therefore, the peptide in peak II is most likely Gly 18−Arg 29 with Cys 25 labeled by iodoacetate. The two peptides in peak IV are GFPTEYF-(CMC)T... and SYVNDVSIH.... While the first peptide was the same as the one in peak II and was also shown to have Cys 25 carboxymethylated by iodoacetate, the second peptide was most likely Ser 109−Lys 131, which does not have cysteine in it (Figure 4) and was thus most likely a contaminant. Therefore, Cys 25 in schistosomal HGPRTase was specifically protected by *P*Rib*PP*−Mg^{2+} against iodoacetate.

DISCUSSION

The results presented demonstrate that GMP-2′,3′-dialdehyde (ox-GMP) labels three sites in Ser 4−Arg 47 and

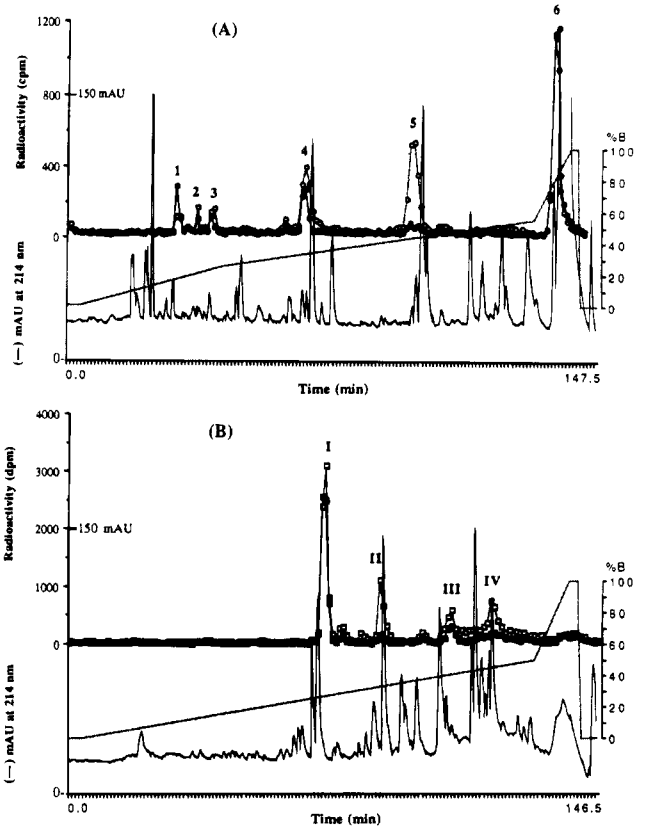

FIGURE 6: Reverse-phase HPLC separation of the tryptic digest of [^{14}C]iodoacetate-labeled human HGPRTase (panel A) and [^{3}H]iodoacetate-labeled schistosomal HGPRTase (panel B). Human HGPRTase (300 μg, 12.5 nmol of the monomer) was labeled with iodoacetate in the absence (○) or presence (●) of 20 mM *P*Rib*PP*, desalted by excessive dialysis, denatured, digested with trypsin, and subjected to HPLC. The peptides were eluted at 1 mL/min with solvent B as follows: a linear gradient of 0−25% over 40 min and then a linear gradient of 25−55% over 90 min followed by a linear gradient of 55−100% over 10 min. Schistosomal HGPRTase (300 μg, 11.5 nmol of the monomer) was labeled with [^{3}H]iodoacetate in the absence (□) or presence (■) of 20 mM *P*Rib*PP*. The elution conditions were as described in Figure 3B. Radioactivity was monitored by counting of 10 μL aliquots from 1 mL fractions.

Ser 91−Arg 100 in human HGPRTase and two sites in Gly 95−Lys 133 in the schistosomal HGPRTase. Ox-GMP labeling of these sites is at random but proceeds at a relatively high rate. Most of the enzyme monomers are each labeled with one ox-GMP within 10 min, which is apparently sufficient to inactivate the enzyme. The presence of one ox-GMP slows down considerably the rates of labeling the other sites, suggesting that these sites are clustered together. Dialdehyde derivatives of nucleotides react with the ϵ-amino group of lysine or the guanidino group of arginine to form either a Schiff base which is stabilized by reduction with sodium borohydride or a dihydroxymorpholino derivative which is unaffected by the reducing agent [see Colman (1990) for review]. Tsai and Hogenkamp (1983) described another possible reaction between the dialdehyde nucleotides (ox-GDP and ox-CDP) and the active site residues of ribonucleotide reductase. They suggested that the enzyme catalyzed the elimination of 5′-pyrophosphate by a nucleophilic attack at C-4′ and that the resulting α,β-unsaturated nucleoside dialdehyde or its corresponding α,β-unsaturated dihydroxymorpholino derivative was attacked by another nucleophilic residue in the active site and became covalently attached to the enzyme. This linkage formation was un-

affected by subsequent reaction with sodium borohydride. The modification of both human and schistosomal HGPRTases with ox-GMP was previously shown to require sodium borohydride reduction to gain stability at acid pH (Kanaaneh et al., 1994). They are thus most likely simple Schiff base formation between the aldehyde groups of ox-GMP and the ϵ-amino group of lysine or the guanidino group of arginine.

Since trypsin still recognizes the ox-GMP−arginine adduct in a peptide (Signor et al., 1971), Arg 33, Arg 44, Arg 47, and Arg 100 in human HGPRTase can be regarded as the most likely sites forming adducts with ox-GMP. However, since only three ox-GMP molecules were bound to one human HGPRTase monomer at a saturating concentration of the former, one of the four arginine residues may not be significantly labeled by ox-GMP. It could be Arg 47, because Val 34−Arg 44 and Val 34−Arg 47 have a similar molar ratio of ox-GMP to peptide of 0.79. (If Arg 47 is labeled similarly, the ratio should double in the second peptide.) It is somewhat less clear which amino acid residue in schistosomal HGPRTase was labeled by ox-GMP because it is not known whether trypsin will recognize ox-GMP−lysine as substrate. Assuming that it will not, Lys 109, Lys 131, and Lys 133 at the C-termini of radiolabeled peptides a, b, and d should not be regarded as the sites of ox-GMP labelings. It leaves then Arg 106 in peptide a as the potential site for ox-GMP labeling. Arg 106 at the C-terminus of peptide c is apparently very poorly labeled by ox-GMP. Because peptide c is much more abundant than peptide a (see Figure 3B), ox-GMP labeling of Arg 106 may be rather limited under the present experimental conditions. The sites for ox-GMP labeling in peptides b and d are not known unless one assumes that ox-GMP−lysine-containing peptide can be digested by trypsin. Lys 131 should be, then, the site for the ox-GMP label. Thus, a tentative conclusion would elucidate that Arg 106 and Lys 131 are labeled by ox-GMP.

The loss of activity of an enzyme by chemical modifications of amino acid residues in the enzyme molecule does not in itself constitute evidence for the location of those residues in the active site. But our previous observations that ox-GMP inactivation of human HGPRTase could be specifically blocked by either *P*Rib*PP* or GMP and only by GMP in schistosomal HGPRTase (Kanaaneh et al., 1994) suggest that ox-GMP binds to the active sites of both enzymes. This conclusion is further supported by the present findings that ox-GMP labeling of the peptides A−C of human HGPRTase can be effectively blocked by *P*Rib*PP* or GMP (Figure 3A) while the labeling of schistosomal HGPRTase can be inhibited by GMP only (data not shown). It is thus likely that peptides Ser 4−Arg 47 and Ser 91−Arg 100 in human HGPRTase are at the binding site(s) for both *P*Rib*PP* and GMP whereas peptide Gly 95−Lys 133 in schistosomal HGPRTase is involved with the GMP-binding site.

It was speculated that a cysteine thiol may be involved in *P*Rib*PP* binding to human HGPRTase on the basis of differences in the K_m values for *P*Rib*PP* between lymphoblast, erythrocyte, and recombinant HGPRTases (Free et al., 1990). The higher K_m for the erythrocyte enzyme was attributed to the oxidation of thiol groups which occurs during aging of the enzyme. Krenitsky and Papaioannou (1969) found that the sulfhydryl reagents *p*-(chloromercuri)-benzoate and *N*-ethylmaleimide inhibited the activity of HGPRTase from human erythrocytes but did not inhibit if the enzyme was treated with *P*Rib*PP*. These studies led Keough et al. (1991) to identify Cys 22 in human HGPRTase to be specifically protected by *P*Rib*PP* against iodoacetate labeling, which was confirmed in our present study. When one compares those findings with the present results from ox-GMP labeling, and the previous observation that ox-GMP inactivation of human HGPRTase can be very effectively blocked by *P*Rib*PP* (Kanaaneh et al., 1994), the data are in good agreement in pointing out that the N-terminal portion of the human enzyme is involved in the binding of *P*Rib*PP* as well as GMP. Our data from studying the schistosomal HGPRTase can render further support to this conclusion in a negative way. The Cys 25 residue in this enzyme is protected by *P*Rib*PP* against carboxymethylation and is thus most likely involved in *P*Rib*PP* binding. However, *P*Rib*PP* is incapable of protecting schistosomal HGPRTase against ox-GMP inactivation or labeling, suggesting that the ox-GMP binding site is separated from the *P*Rib*PP* binding site (Kanaaneh et al., 1994). This postulation is well supported by our present finding that ox-GMP does not label the N-terminal portion of schistosomal enzyme at all but, instead, labels probably Arg 106 and Lys 131. This distinction between human and schistosomal HGPRTases should be further exploited for potential opportunities for antischistosomal chemotherapy.

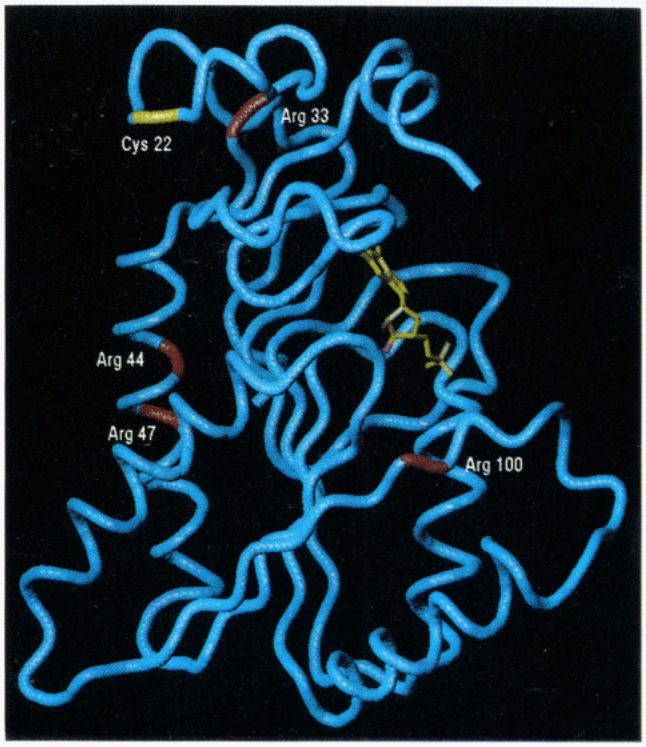

FIGURE 7: C_α schematic of the human HGPRTase monomer with GMP bound at the active site. Cys 22 is shown in yellow, and the ox-GMP-modified arginines are shown in red. The 2′- and 3′-hydroxyls of GMP are shown in pink. The coordinates of human HGPRTase were kindly provided by J. C. Eads and J. C. Sacchettini of Albert Einstein College of Medicine.

When the biochemical information is correlated to the crystal structure of the human HGPRTase−GMP complex (Eads et al., 1994), the locations of the two ox-GMP-labeled peptides and of Cys 22 are all far removed from the apparent binding site of GMP in the crystal structure (Figure 7). The sulfur atom of Cys 22 is 26−27 Å away from the ribose

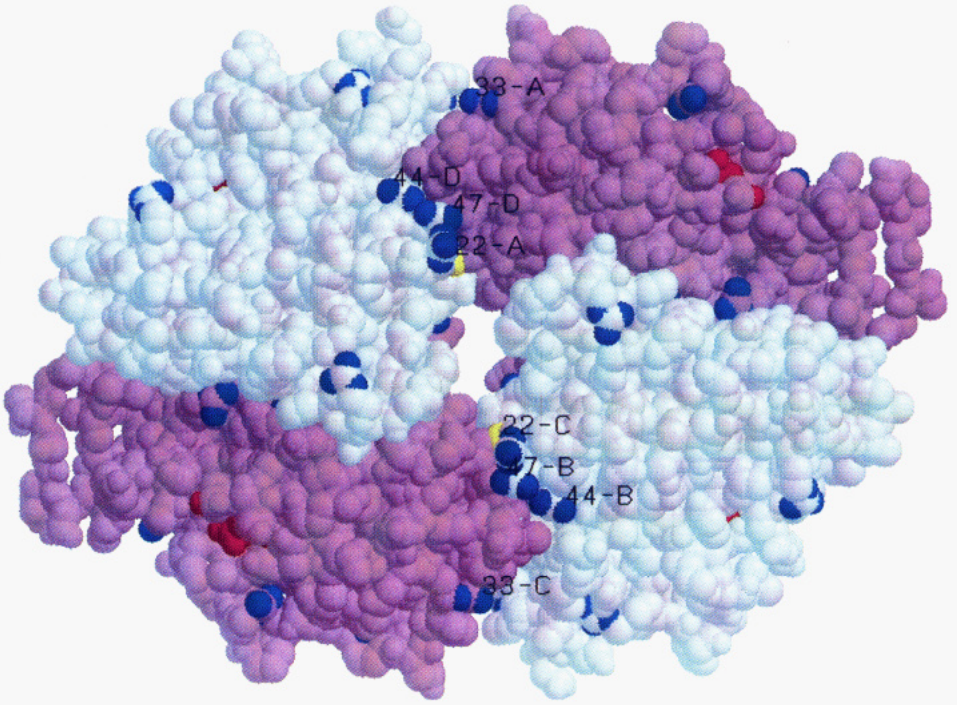

FIGURE 8: Locations of the ox-GMP-modified arginines and Cys 22 in the human HGPRTase tetramer. The relationship of the monomer to the tetramer can be described by three perpendicular 180° rotations. The first 2-fold operation generates the dimer from the monomer, while another 2-fold operation generates the tetramer from two dimers. Two orthogonal 2-fold axes are in the plane of the figure in the vertical and horizontal directions, and the third 2-fold axis is perpendicular to the figure. Cys 22 is shown in yellow, arginine nitrogens are in blue, and GMP is shown in red. Arg 33, Arg 44, and Arg 47 form a cluster in the vicinity of Cys 22. The four different monomers are designated A−D.

oxygens O2′ and O3′ of bound GMP. The average distance between the NH_2 of the guanidino groups of each of the two arginines, Arg 33 and Arg 44, and the ribose O2′ and O3′ of GMP is approximately 25 Å. Arg 100 lies closer to the GMP than the other two arginines with an average distance of 10 Å between the NH_2 of its guanidino group and the ribose oxygens of GMP. These results indicate that both ox-GMP and *P*Rib*PP* bind to residues that are far removed from the proposed active site depicted in the crystal structure (Figure 7). The only way that Arg 33, Arg 44, and Cys 22 could participate in the binding of GMP and *P*Rib*PP* is to have the amino terminus undergo a major conformational change upon binding of *P*Rib*PP*. Such a conformational change would require a major positional shift of an N-terminal ~50 amino acid segment of the protein, consisting of two β-sheets and two α-helical structures, to move by a distance of about 25 Å upon the binding of *P*Rib*PP* to Cys 22. The end result would be a conformation similar to that of the OPRTase crystal (Scapin et al., 1994) in which the N-terminus is very close to the active site, allowing effective *P*Rib*PP* protection of Lys 26 (Grubmeyer et al., 1993). This conformational change is not entirely impossible in view of the fact that human HGPRTase catalyzes an ordered bi-bi random bi-bi reaction in which *P*Rib*PP*−Mg^{2+} must bind to the enzyme first before hypoxanthine or guanine can bind to the enzyme to initiate the reaction (Giacomello & Salerno, 1978). The binding of *P*Rib*PP*−Mg^{2+} to human HGPRTase is also known to greatly stabilize the enzyme (Yuan et al., 1993), suggesting that *P*Rib*PP* binding may lock the enzyme in a stable and active state. However, if one accepts the current crystal structure of the human HGPRTase−GMP complex (Eads et al., 1994) as another natural state of the enzyme during the enzyme-catalyzed reaction, the oscillation between it and another conformation resembling that of OPRTase would be too extensive a conformational change for a rapid enzymatic reaction with an estimated k_{cat} value of 33 s^{-1} (Bhatia et al., 1990). The flip-flops between two drastically different conformations would be physically and thermodynamically infeasible. Then, the only plausible explanation left would be that the current crystal structure of the human HGPRTase−GMP complex may represent an inactive form. Such a possibility can be examined by data from the HGPRTase−*P*Rib*PP* crystals.

Human HGPRTase has been reported to exist in its active state as a dimer or as a tetramer, depending on the ionic strength and pH, even though there is no evidence to suggest that the monomer in its native state is necessarily enzymatically inactive. *P*Rib*PP* and GMP have been shown to catalyze the formation of tetramers (Johnson et al., 1979; Strauss et al., 1978; Free et al., 1990). Under the conditions used for ox-GMP labeling in this report, the human HGPRTase was determined by gel filtration to exist as a mixture of dimer and tetramer (data not shown). Although the locations of arginine residues Arg 33, Arg 44 and Arg 47 in the crystal structure of the human HGPRTase monomer are relatively distant from one another and Cys 22, they form a cluster in the vicinity of Cys 22 at the interface of two monomers in the tetramer (Figure 8). Thus, the cluster could attract the binding of ox-GMP, but it cannot explain the specificity and the inhibitory effect of the ox-GMP action unless one assumes that the clusters function also as allosteric sites. Although Craft et al. (1970) reported on the allosteric properties for the human HGPRTase, other workers who later studied the steady-state kinetics of the human HGPRTase did not observe such an effect (Giacomello & Salerno, 1978, 1981). A crystal structure of human HGPRTase complexed with *P*Rib*PP* and Mg^{2+} will elucidate additional details on the binding of Mg^{2+}−*P*Rib*PP*. Whether the identified

arginine residues and Cys 22 are essential to the enzyme activity remains an important question which is currently being probed in our laboratory by means of site-directed mutagenesis.

The crystal structure for the schistosomal HGPRTase is not yet available. However, in the primary structure of the schistosomal HGPRTase the likely ox-GMP-modified residues, Arg 106 and Lys 131, are located close to the putative motif binding to the 5′-phosphate moiety of *P*Rib*PP* consisting of the residues Val 135−Thr 147 and away from the N-terminal portion of the protein. The labeling of the schistosomal HGPRTase with ox-GMP was inhibited by GMP but not by *P*Rib*PP*, while the latter was shown to protect Cys 25 against iodoacetate labeling. Together, these results suggest that either the active pocket in the schistosomal HGPRTase is less compact than the human HGPRTase, so it can accommodate both ox-GMP and *P*Rib*PP*, or that each substrate binds to an independent active pocket. Further studies by site-directed mutagenesis should be able to answer some of these uncertainties.

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REFERENCES

Argos, P., Hanei, M., Wilson, J. M., & Kelley, W. N. (1983) *J. Biol. Chem. 258*, 6450−6457.
Bhatia, M. B., Vinitsky, A., & Grubmeyer, C. (1990) *Biochemistry 29*, 10480−10487.
Colman, R. F. (1990) *Enzymes 19*, 287−321.
Craft, J. A., Dean, B. M., Watts, R. W. E., & Westwick, W. J. (1970) *Eur. J. Biochem. 15*, 367−373.
Craig, S. P., III, McKerrow, J. H., Newport, G. R., & Wang, C. C. (1988) *Nucleic Acids Res. 16*, 7087−7101.
Craig, S. P., III, Yuan, L., Kuntz, D. A., McKerrow, J. H., & Wang, C. C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2500−2504.
Davidson, B. L., Brown, J. E., Weber, C. H., Palella, T. D., & Roessler, B. J. (1993) *Gene 123*, 271−275.
Dovey, H. F., McKerrow, J. H., & Wang, C. C. (1984) *Mol. Biochem. Parasitol. 11*, 157−167.
Eads, J. C., Scapin, G., Xu, Y., Grubmeyer, C., & Sacchetteni, J. C. (1994) *Cell 78*, 325−334.
Free, M. L., Gordon, R. B., Keough, D. T., Beacham, I. R., Emmerson, B. T., & de Jersey, J. (1990) *Biochim. Biophys. Acta 1087*, 205−211.
Giacomello, A., & Salerno, C. (1978) *J. Biol. Chem. 253*, 6038−6044.
Giacomello, A., & Salerno, C. (1981) *J. Biol. Chem. 256*, 3671−3673.
Grubmeyer, C., Segura, E., & Dorfman, R. (1993) *J. Biol. Chem. 268*, 20299−20304.
Hountondji, C., Schmitter, J. M., Beauvallet, C., & Blanquet, S. (1990) *Biochemistry 29*, 8190−8198.
Hove-Jensen, B., Harlow, K. W., King, C. J., & Switzer, R. L. (1986) *J. Biol. Chem. 261*, 6765−6771.
Johnson, G. G., Eisenberg, L. R., & Migeon, B. R. (1979) *Science 203*, 174−176.
Jolly, D. J., Okayama, H., Berg, P., Esty, A. C., Filpula, D., Bohlen, P., Johnson, G. G., Shively, J. E., Hunkapillar, T., & Friedman, T. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 477−481.
Kanaaneh, J., Craig, S. P., III, & Wang, C. C. (1994) *Eur. J. Biochem. 223*, 595−601.
Kelley, W. N., Rosenbloom, F. M., Henderson, J. F., & Seegmiller, J. E. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 1735−1739.
Keough, D. T., Emmerson, B. T., & de Jersey, J. (1991) *Biochim. Biophys. Acta 1096*, 95−100.
King, T. P. (1966) *Biochemistry 5*, 3454−3459.
Krenitsky, T. A., & Papaioannou, R. (1969) *J. Biol. Chem. 244*, 1271−1277.
Scapin, G., Grubmeyer, C., & Sacchettini, J. C. (1994) *Biochemistry 33*, 1287−1294.
Seegmiller, J. E., Rosenbloom, F. M., & Kelley, W. N. (1967) *Science 155*, 1682−1684.
Senft, A. W., & Crabtree, G. W. (1983) *Pharmacol. Ther. 20*, 341−356.
Signor, A., Bonora, G. M., Biondi, L., Nisato, D., & Marzotto, A. (1971) *Biochemistry 10*, 2748−2752.
Smith, J. L., Zaluzec, E. J., Wery, J., Niu, L., Switzer, R. L., Zalkin, H., & Satow, Y. (1994) *Science 264*, 1427−1433.
Strauss, M., Behlke, J., Ampers, F., & Goerl, M. (1978) *Eur. J. Biochem. 90*, 89−97.
Tsai, P. K., & Hogenkamp, H. P. C. (1983) *Arch. Biochem. Biophys. 226*, 276−284.
Wilson, J. M., Young, A. B., & Kelly, W. N. (1983) *N. Engl. J. Med. 309*, 900−910.
Yuan, L., Craig, S. P., III, McKerrow, J. H., & Wang, C. C. (1990) *J. Biol. Chem. 265*, 13528−12532.
Yuan, L., Craig, S. P., III, McKerrow, J. H., & Wang, C. C. (1992) *Biochemistry 31*, 806−810.
Yuan, L., Wu, C.-S. C., Craig, S. P., III, Liu, A. F., & Wang, C. C. (1993) *Biochim. Biophys. Acta 1162*, 10−16.

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