Characterization of Two Forms of Poly(ADP-ribose) Glycohydrolase in Guinea Pig Liver†

Hideharu Maruta, Kiyoshi Inageda, Tsutomu Aoki, Hiroshi Nishina, and Sei-ichi Tanuma*

Department of Life Science, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 227, Japan

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ABSTRACT: A poly(ADP-ribose) glycohydrolase from guinea pig liver cytoplasm has been purified approximately 45 000-fold to apparent homogeneity. The cytoplasmic poly(ADP-ribose) glycohydrolase designated form II differed in several respects from the nuclear poly(ADP-ribose) glycohydrolase I (M_r = 75 500) previously purified from the same tissue (Tanuma et al., 1986a). The purified glycohydrolase II consists of a single polypeptide with M_r of 59 500 estimated by a sodium dodecyl sulfate–polyacrylamide gel. A native M_r of 57 000 was determined by gel permeation. Peptide analysis of partial proteolytic degradation of glycohydrolases II and I with *Staphylococcus aureus* V8 protease revealed that the two enzymes were structurally different. Amino acid analysis showed that glycohydrolase II had a relatively low proportion of basic amino acid residues as compared with glycohydrolase I. Glycohydrolase II and I were acidic proteins with isoelectric points of 6.2 and 6.6, respectively. The optimum pH for glycohydrolases II and I were around 7.4 and 7.0, respectively. The K_m value for $(ADP\text{-}ribose)_n$ (average chain length n = 15) and the V_{max} for glycohydrolase II were 4.8 μM and 18 μmol of ADP-ribose released from $(ADP\text{-}ribose)_n \cdot min^{-1} \cdot (mg\ of\ protein)^{-1}$, respectively. The K_m was about 2.5 times higher, and V_{max}, 2 times lower, than those observed with glycohydrolase I. Unlike glycohydrolase I, glycohydrolase II was inhibited by monovalent salts. ADP-ribose and cAMP inhibited glycohydrolase II more strongly than glycohydrolase I. These results suggest that eukaryotic cells contain two distinct forms of poly(ADP-ribose) glycohydrolase exhibiting differences in properties and subcellular localization.

Poly(ADP-ribosylation) is generally thought to be a nuclear event in eukaryotic cells. Several lines of evidence support the idea that poly(ADP-ribosylation) of chromosomal proteins plays regulatory roles in DNA repair, replication, and transcription, and thereby in the cell cycle and differentiation (Ueda & Hayaishi, 1985; Sugimura & Miwa, 1983; Mandel et al., 1982). However, the true physiological function of this modification has not yet been established, since it is difficult to carry out in vivo experiments needed to progress beyond such vague suggestions of the biological significance of protein poly(ADP-ribosylation).

Our approach to better understanding of the physiological significance of poly(ADP-ribosylation) is to analyze enzymes that participate in $(ADP\text{-}ribose)_n$ metabolism. An enzyme, poly(ADP-ribose) synthetase, that is responsible for the synthetic reactions, initiation, and elongation of $(ADP\text{-}ribose)_n$ has been examined well (Okayama et al., 1977; Yoshihara et al., 1978; Ito et al., 1979). On the other hand, two types of poly(ADP-ribose)-degrading enzymes have been found and purified. One enzyme, poly(ADP-ribose) glycohydrolase, catalyzes hydrolysis of the glycosidic (1″–2′) linkages of $(ADP\text{-}ribose)_n$ (Miwa et al., 1974; Tavassoli et al., 1983; Tanuma et al., 1986a–c; Hatakeyama et al., 1986; Tanuma & Endo, 1990a; Thomassin et al., 1990). A second type is ADP-ribose–protein lyase, which is capable of splitting only mono(ADP-ribose)–protein linkages (Oka et al., 1984). These two enzymes have been thought to be involved in de-poly(ADP-ribosylation) of chromosomal proteins.

Previously, we first purified poly(ADP-ribose) glycohydrolase to homogeneity from nuclei of guinea pig liver (Tanuma et al., 1986a). In the course of our study on the purification of poly(ADP-ribose) glycohydrolase, we found the presence of a poly(ADP-ribose) glycohydrolase in cytoplasmic fractions of guinea pig liver, HeLa S3 cells, and human erythrocytes and suggested the possible involvement in extranuclear de-poly(ADP-ribosylation) (Tanuma et al., 1986a–c; Tanuma & Endo, 1990a). In this report, we extend the previous work and achieve purification of cytoplasmic poly(ADP-ribose) glycohydrolase from guinea pig liver to homogeneity, and the two forms of poly(ADP-ribose) glycohydrolase were biochemically characterized. To distinguish the cytoplasmic glycohydrolase from the nuclear glycohydrolase previously purified from the same tissue (Tanuma et al., 1986a), the cytoplasmic enzyme described herein is designated poly(ADP-ribose) glycohydrolase II, and the nuclear enzyme, poly(ADP-ribose) glycohydrolase I.

Experimental Procedures

Materials

[*adenine*-2,8-^{3}H]NAD (27 Ci/mmol) was purchased from Du Pont–New England Nuclear. DEAE-cellulose (DE-52), phosphocellulose (P-11), and DE-81 paper were from Whatman. Blue Sepharose and Red Sepharose were from Pharmacia LKB Biotechnology Inc. Single-stranded DNA cellulose was from Sigma. TSK-G2000SW and phenyl-5PWRP were from Tosoh LTD., Japan. *Staphylococcus aureus* V8 protease was from Miles Laboratories Inc.

Methods

Preparation of $(ADP\text{-}ribose)_n$. Isolation of varying chain size (ADP-ribose residues per polymer molecule) was performed with DEAE-cellulose column chromatography (Kawaichi et al., 1981). The average chain size, average chain length (ADP-ribose residues per branch of polymer molecule),

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* To whom correspondence should be addressed.

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and average branching point (number of branching points per polymer molecule) were calculated from the data of phosphodiesterase digestion as follows (Tanaka et al., 1977; Miwa & Sugimura, 1982):[1]

$$\text{av chain length} = \frac{[5'\text{-AMP}] + [\text{PR-AMP}]}{[5'\text{-AMP}]}$$

$$\text{av chain size} = \frac{[5'\text{AMP}] + [\text{PR-AMP}] + [(\text{PR})_2\text{-AMP}]}{[5'\text{-AMP}] - [(\text{PR})_2\text{-AMP}]}$$

$$\text{av branching point} = \frac{[(\text{PR})_2\text{-AMP}]}{[5'\text{-AMP}] - [(\text{PR})_2\text{-AMP}]}$$

Assay of Poly(ADP-ribose) Glycohydrolase. Poly(ADP-ribose) glycohydrolase activity was measured as previously described (Tanuma et al., 1986a–c). The standard reaction mixture contained 50 mM potassium phosphate (pH 7.4), 10 mM 2-mercaptoethanol, 0.1 mM PMSF, 100 μg/mL bovine serum albumin, 10 μM [^{3}H](ADP-ribose)$_n$ [(2.5–3.0) × 10^6 cpm/μmol of ADP-ribose residues, average chain length of 15]. The reaction was carried out at 37 °C for 10 min. Enzyme activity was determined by measuring the radioactivity of 10% trichloroacetic acid soluble material (assay 1, total volume of 150 μL) or by disappearance of (ADP-ribose)$_n$, which was estimated as DE-81 paper adsorbed radioactivity (assay 2, a total volume of 30 μL). One enzyme unit is defined as the activity to liberate 1 μmol of ADP-ribose from (ADP-ribose)$_n$ per minute under these conditions.

Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide slab gel electrophoresis was performed as described previously using 0.1% SDS and 12.5% acrylamide as described (Laemmli, 1970). The sample was treated at 95 °C for 3 min in 62.5 mM Tris-HCl (pH 6.8), 1% SDS, 2% 2-mercaptoethanol, and 10% glycerol and then loaded onto polyacrylamide slab gels. Electrophoresis was carried out at 90 V until bromophenol blue dye migrated about 90% the length of the gel. Protein bands were detected by using silver-stain kit (Dai-ich Kagaku, Tokyo).

Isoelectric Focusing. The isoelectric point of poly(ADP-ribose) glycohydrolase was determined in Pharmacia Phast System using Phast Gel IEF (pH 3–9).

Analysis of Amino Acid Composition. Amino acid analysis of purified enzyme was determined by hydrolysis with 6 N HCl at 110 °C for 24, 48, and 72 h followed by analysis on a Hitachi amino acid analyzer, Model 835 (Spademan et al., 1958). Serine and threonine contents were determined by extrapolation to zero time of the three points.

Analysis of Degradation Product of (ADP-ribose)$_n$. Samples were spotted on a cellulose thin-layer plate and developed with mobile phase consisting of 0.1 M sodium phosphate buffer (pH 6.8), ammonium sulfate, and 1-propanol (100:60:2 v/w/v) at room temperature (Tanuma et al., 1986a–c). The thin-layer plate was cut into pieces (0.75-cm width), and radioactivity was counted in a liquid scintillation counter. Sample were also analyzed by strong anion exchange (Hitachi CHI gel no. 3013-N) HPLC. Material was eluted with a linear gradient from solvent A (1.5% acetonitrile, 15 mM ammonium chloride, 2.5 mM potassium phosphate monobasic, and 2.5 mM potassium phosphate dibasic) to solvent B (6% acetonitrile, 300 mM ammonium chloride, 50 mM potassium phosphate monobasic, and 50 mM potassium phosphate dibasic) (Tanuma & Endo, 1990b).

Other Methods. Protein concentration was measured with bovine serum albumin as the standard (Lowry et al., 1951).

Purification of Poly(ADP-ribose) Glycohydrolase II. All the operations were performed at 0–4 °C unless otherwise indicated. To avoid freezing, potassium phosphate buffer used in the purification was supplemented with 30% ethylene glycol. Buffers used were buffer A [10 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 1 mM Na_2EDTA, 2 mM 2-mercaptoethanol, and 0.3 mM PMSF] and buffer E [50 mM potassium phosphate (pH 7.2), 10 mM 2-mercaptoethanol, 0.3 mM PMSF, and 30% ethylene glycol].

Step 1: Cytoplasm. Guinea pig liver (wet weight 500 g) was homogenized in 3 volumes of 0.25 M sucrose–buffer A with a Waring Blender for 15 s × 2. The homogenate was filtered through a stainless mesh (100 μm), yielding the cell homogenate. The homogenate was layered onto 2.2 M sucrose–buffer A and separated into nuclear and cytoplasmic fractions by centrifugation at 40000*g* for 60 min (fraction I).

Step 2: Cytosol. The cytoplasmic fraction (fraction I) was further centrifuged at 105000*g* for 60 min to obtain cytosolic fraction (fraction II).

Step 3: Phosphocellulose Column. The cytosol was adjusted to 0.1 M KCl and stirred with a 50-mL bed volume of phosphocellulose for 1 h. The phosphocellulose was allowed to settle and washed twice with 0.1 M KCl–buffer E. The washed resin was then transferred into a column, and proteins were eluted from the column with a linear gradient formed from 0.1 to 1 M KCl–buffer E. Fractions containing (ADP-ribose)$_n$-degrading enzyme activity eluted at 0.28 M KCl were pooled and dialyzed against buffer E (fraction III).

Step 4: DEAE-cellulose Column. Fraction III was applied to a column of DEAE-cellulose (1.5 × 15 cm) previously equilibrated with buffer E. The column was washed with 2 column volumes of the same buffer, and proteins were eluted with a linear gradient of KCl (0–1 M) in buffer E. The active fractions eluting at 0.18 M KCl were pooled (fraction IV).

Step 5: Blue Sepharose. Fraction IV was applied to a Blue Sepharose column (1.0 × 10 cm) previously equilibrated with buffer E. The column was washed with 2 column volumes of the same buffer, and proteins were eluted with a linear gradient of KCl (0–1 M) in buffer E. The active fractions eluted at 0.33 M KCl were pooled and dialyzed against buffer E (fraction V).

Step 6: Single-Stranded DNA Cellulose. The dialyzed sample was applied to a single-stranded DNA cellulose column (0.7 × 13 cm) previously equilibrated with buffer E. Proteins were eluted with a linear gradient of KCl (0–1 M) in buffer E. The activity of poly(ADP-ribose) glycohydrolase II was eluted at 0.24 M KCl (fraction VI).

Step 7: Red Sepharose Column. Fraction VI was applied to a column of Red Sepharose (0.7 × 8 cm) and the enzyme was eluted with a linear gradient of KCl (0 to 2 M) in buffer E. The active fraction eluted at 0.56 M KCl was pooled (fraction VII).

Step 8: TSK-G2000SW Column. Fraction VII was applied to a TSK-G2000SW column (0.75 × 30 cm) preequilibrated with buffer E containing 1 M KCl. Elution was carried out with the same buffer at flow rate of 0.5 mL/min. β-Amylase (200 000), alcohol dehydrogenase (150 000), bovine serum albumin (66 000), carbonic anhydrase (29 000), and cytochrome *c* (12 400) were used as molecular weight standards. The enzyme fractions were pooled and dialyzed against buffer E (fraction VIII).

[1] Abbreviations: SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; PR-AMP, 2′-(5″-phosphoribosyl)-5′-AMP; (PR)$_2$-AMP, 2′-[2″-(1‴-ribosyl)-1″-ribosyl]adenosine 5′,5″,5‴-trisphosphate; HPLC, high-performance liquid chromatography.

Table I: Purification of Poly(ADP-ribose) Glycohydrolase II from Guinea Pig Liver Cytoplasm[a]

purification step	protein (mg)	act. (μmol·min^{-1})	sp act. (μmol·min^{-1}·mg^{-1})	yield (%)	purification (x-fold)
(1) cytoplasm	29 800	10.2	0.0003	100	1
(2) cytosol	8 770	9.61	0.0011	94.2	3.7
(3) phosphocellulose	281	7.82	0.028	76.7	93.3
(4) DEAE-cellulose	50.1	6.83	0.136	67.0	453
(5) Blue Sepharose	6.87	4.32	0.629	42.4	1 410
(6) single-stranded DNA cellulose	1.30	3.47	2.67	34.0	8 900
(7) Red Sepharose	0.381	2.82	7.40	26.3	24 700
(8) TSK-G2000SW	0.114	1.53	13.4	15.0	44 700

[a] The poly(ADP-ribose) glycohydrolase II activity was measured according to assay 1 as described under Experimental Procedures.

For analysis of the amino acid composition of poly(ADP-ribose) glycohydrolase, fraction VIII was further purified by reverse-phase HPLC on a phenyl-5PWRP column (0.5 × 0.5 cm). The column was washed with 5% acetonitrile–0.05% trifluoroacetic acid solution, and then proteins were eluted with a linear gradient of 7–70% acetonitrile in 0.05% trifluoroacetic acid solution.

Results

Purification of Cytoplasmic Poly(ADP-ribose) Glycohydrolase II. Our previous study of the distribution of poly(ADP-ribose) glycohydrolase in guinea pig liver has shown that about 20% of the total activity of the homogenate was present in the cytoplasm (Tanuma et al., 1986a). Almost all of the cytoplasmic poly(ADP-ribose) glycohydrolase activity was recovered in the cytosolic fraction (Table I). Thus, we attempted to purify the cytoplasmic poly(ADP-ribose) glycohydrolase from the cytosol to homogeneity by a modification of the procedure used previously (Tanuma et al., 1986a). Table I shows a summary of the cytoplasmic poly(ADP-ribose) glycohydrolase II purification procedure. The enzyme was purified about 45 000-fold from the cytoplasm, with a yield of 15%. The specific activity of the final fraction was free of phosphodiesterase and nicotinamide adenine dinucleotidase activities. The enzyme was routinely stored at −20 °C in buffer E. Under these conditions, the catalytic activity was very stable for at least 6 months.

Physical Properties of Poly(ADP-ribose) Glycohydrolase II. (*A*) *Molecular Weight.* On SDS–polyacrylamide gel electrophoresis, the purified poly(ADP-ribose) glycohydrolase II migrated as a single protein band with a polypeptide molecular weight of 59 500 (Figure 1). The native molecular weight of glycohydrolase II was determined by gel permeation of TSKG-2000SW HPLC. The glycohydrolase II activity was eluted from the column as a single peak at a position between bovine serum albumin and ovalbumin markers. The apparent molecular weight of glycohydrolase II was estimated to be 57 000. These results indicated that the enzyme was composed of a monomeric polypeptide. It appeared that the molecular weight of glycohydrolase II was much lower than that of glycohydrolase I, which had a molecular weight of 75 500 (Tanuma et al., 1986a).

(*B*) *Peptide Mapping.* To test the structural difference of glycohydrolases II and I, their proteolytic fragments by *S. aureus* V8 protease were compared. As shown in Figure 1, peptides derived from glycohydrolase II contained two main bands (lanes b and c), whereas the peptide map of glycohydrolase I indicated two (lane e) or three (lane d) major bands. These bands were barely distinguishable.

(*C*) *Isoelectric Focusing.* The p*I* value of poly(ADP-ribose) glycohydrolases II and I were estimated on a isoelectric focusing system based on the mobilities of p*I* standards. Glycohydrolases II and I were found to be acidic proteins having p*I* values of 6.2 and 6.6, respectively.

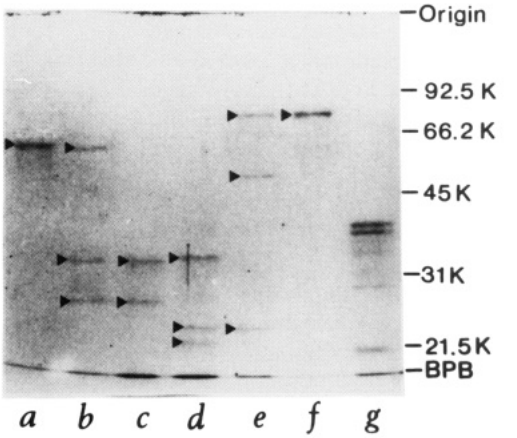

FIGURE 1: Determination of molecular weight of the purified glycohydrolase II and peptide mapping of glycohydrolases II and I with *S. aureus* V8 protease. Partial proteolysis with *S. aureus* V8 protease was done according to Cleaveland et al. (1977). About 300 ng of glycohydrolase II (lanes a–c) or I (lanes d–f) was treated at 100 °C in SDS sample buffer and then loaded onto 0.1% SDS and 12.5% polyacrylamide gels with 0 (a, f), 0.3 (b, e), and 3 (c, d) ng of the V8 protease. Proteins were partially digested during electrophoresis. Lane g contained 500 ng of the protease alone. The molecular weight standards used were as follows: phosphorylase *b* (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), and soybean trypsin inhibitor (21 500). Positions of proteolytic fragments detected by silver staining are indicated by solid arrowheads.

Table II: Amino Acid Composition of Poly(ADP-ribose) Glycohydrolases I and II

amino acid	glycohydrolase I (mol/100 mol)[a]	glycohydrolase II (mol/100 mol)[a]
Asx	8.92	9.64
Thr	5.01	5.18
Ser	9.20	11.84
Glx	11.2	13.3
Gly	11.3	13.1
Ala	9.41	9.36
Cys	nd[b]	nd
Val	6.02	6.84
Met	1.88	1.94
Ile	3.56	3.07
Leu	9.11	6.28
Tyr	2.63	3.01
Phe	3.41	3.45
Lys	6.58	2.65
His	2.79	2.91
Arg	6.69	2.38
Pro	2.20	5.01
basic[c]	16.1	7.94
acidic[d]	20.2	22.9
acidic/basic	1.26	2.88

[a] The values were averages from the analyses performed after 24-, 48-, and 72-h acidic hydrolysis. [b] nd = not detected. [c] Basic = sum of Lys, His, and Arg contents. [d] Acidic = sum of Asx and Glx contents.

(*D*) *Amino Acid Composition.* The results of amino acid composition of the purified glycohydrolases II and I are sum-

Table III: Effect of Various Chain Lengths of (ADP-ribose)$_n$ on Poly(ADP-ribose) Glycohydrolases I and II[a]

(ADP-ribose)$_n$			glycohydrolase I		glycohydrolase II	
av chain length (n)	av chain size	av branching point	K_m (μM)	V_{max} (μmol·min^{-1}·mg^{-1})	K_m (μM)	V_{max} (μmol·min^{-1}·mg^{-1})
6.7	7.1	0.05	4.5	23	6.6	11
15	16	0.10	2.0	43	4.8	18
21	28	0.31	1.1	56	4.0	23
27	77	1.8	0.58	78	3.1	31

[a] The K_m and V_{max} values were determined by Lineweaver–Burk reciprocal plots. Five different substrate concentrations were used for the determination of each K_m and V_{max} value. At each concentration, activity was determined in duplicate by assay 2. The values are the average of two independent determinations. The two values agreed within 6–11% of each other.

marized in Table II. This analysis showed glycohydrolase II to have relatively low contents of basic amino acid residues (Lys and Arg). In contrast, glycohydrolase I contained a relatively high proportion of Lys and Arg residues. The basics (sum of Lys, His, and Arg) of glycohydrolases II and I were 7.94 and 16.1, respectively. The ratios of acidic to basic amino acids of glycohydrolases II and I were 1.26 and 2.88, respectively.

Catalytic Properties of Poly(ADP-ribose) Glycohydrolase II. (*A*) *Optimum pH.* The optimum pH of glycohydrolase II activity was around 7.4. This value was higher than the optimum pH 7.0 of the nuclear glycohydrolase I previously purified. In both glycohydrolases, Tris buffer was inhibitory as compared with phosphate buffer. The optimal pH for the enzyme stability was broad, in the range between 6 and 9.

(*B*) *Requirement of Sulfhydryl Compound.* Both glycohydrolases II and I showed a similar sulfhydryl compound requirement; the glycohyrolase II activity was stimulated about 2-fold by 10 mM 2-mercaptoethanol or 5 mM dithiothreitol.

(*C*) *Mode of Hydrolysis.* The hydrolytic product of (ADP-ribose)$_n$ with glycohydrolase II, which was analyzed by cellulose thin-layer chromatography and HPLC on a strong anion exchange column, had a mobility identical with that of an authentic ADP-ribose marker. Like glycohydrolase I (Tanuma et al., 1986a), in the initial stage of reaction, oligo(ADP-ribose)$_n$ was not observed, suggesting that glycohydrolase II hydrolyzes (ADP-ribose)$_n$ exoglycosidically.

(*D*) *Kinetic Constants.* Under standard assay conditions, the apparent K_m value for (ADP-ribose)$_n$ (average chain length of 15) of glycohydrolase II obtained from a double-reciprocal plot was 4.8 μM. The V_{max} for hydrolysis of (ADP-ribose)$_n$ was calculated to be 18 μmol·min^{-1}·(mg of protein)$^{-1}$. The K_m was about 2.5 times higher, and the V_{max}, 2 times lower, than those of glycohydrolase I [K_m = 2.0 μM and V_{max} = 43 μmol·min^{-1}·(mg of protein)$^{-1}$] (Table I) (Tanuma et al., 1986a). Both enzymes showed variable activities that depended on the chain length of (ADP-ribose)$_n$ (Table III). In glycohydrolase I, the K_m value for a shorter polymer (n = 6.7) was approximately 1 order of magnitude higher than that for a longer polymer (n = 27). On the other hand, the differences in K_m and V_{max} values of glycohydrolase II for shorter and longer polymers were much smaller than those of glycohydrolase I.

(*E*) *Effect of Mono- and Divalent Cations.* Unlike glycohydrolase I (Tanuma et al., 1986a), which was stimulated by low concentrations (50–100 mM) of KCl or NaCl, glycohydrolase II was rather inhibited by the salts (Figure 2). Fifty percent inhibition occurred at 100 mM KCl for glycohydrolase II. At concentrations up to 3 mM, $MgCl_2$, $CaCl_2$, and $MnCl_2$ had little effect on either enzyme.

(*F*) *Effect of DNA.* Heat-denatured DNA inhibited glycohydrolase II more weakly than glycohydrolase I. Heat-denatured DNA inhibited glycohydrolase I by 50% at a concentration of 40 μg/mL. The effect on glycohydrolase II was about 1 order of magnitude less than on glycohydrolase I.

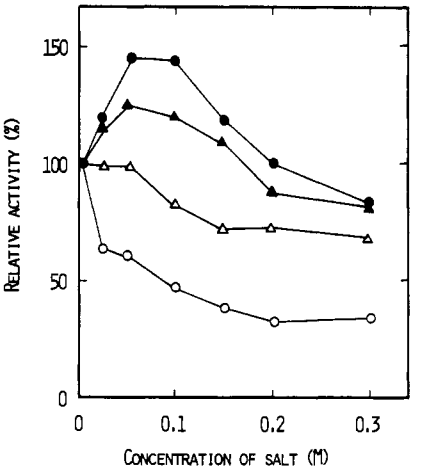

FIGURE 2: Effect of monovalent salts on poly(ADP-ribose) glycohydrolases II and I. The reaction was carried out under the standard conditions except that KCl (●, ○) or NaCl (▲, △) was added at the indicated concentrations. The enzyme activities in no salt are taken as 100%. Glycohydrolase II (○, △); glycohydrolase I (●, ▲). The values were means of triplicate experiments.

Table IV: Effect of Nucleotides on Poly(ADP-ribose) Glycohydrolases I and II[a]

	K_i (±SD) (μM)	
compound	glycohydrolase I	glycohydrolase II
ADP-ribose	480 (±25)	350 (±31)
cAMP	2800 (±84)	1800 (±87)

[a] Inhibition constants (K_i) were determined by using Dixon plots. Three different inhibitor concentrations were used for the determination of each K_i value. At each inhibitor concentration, activity was determined by using four different substrate (ADP-ribose)$_n$ concentrations (each point in duplicate). The best straight line was then determined by linear regression. Results are expressed as means ±SD from three separate experiments.

Native DNA had essentially no effect on either enzyme.

(*G*) *Effect of Nucleotides.* ADP-ribose and cAMP inhibited glycohydrolase II more strongly than glycohydrolase I. ADP, AMP, ATP, and NAD^+ had no inhibitory effect on either enzyme. Further kinetic studies were carried out to characterize the inhibitory action of ADP-ribose and cAMP. The K_i values with ADP-ribose and cAMP were calculated to be 0.35 and 1.8 mM, and 0.48 and 2.8 mM for the reaction of glycohydrolase II and I, respectively (Table IV). These compounds were competitive inhibitors of both glycohydrolase II and I reaction.

Discussion

The cytoplasmic poly(ADP-ribose) exoglycohydrolase from guinea pig liver was purified to apparent homogeneity and characterized. This represents the only comparative study to date between these homogeneous nuclear and cytoplasmic glycohydrolases. Many of the general properties of the cytoplasmic poly(ADP-ribose) glycohydrolase (form II) are

different from those of the nuclear poly(ADP-ribose) glycohydrolase (form I) previously purified (Tanuma et al., 1986a). Poly(ADP-ribose) glycohydrolase I is tightly bound to some chromatin structure, presumably DNA in the nucleus (Tanuma et al., 1986a,b), while poly(ADP-ribose) glycohydrolase II is probably localized in the cytosol (Tanuma et al., 1986c; Tanuma & Endo, 1990a). In addition to their possibly different intracellular localization, the two forms were markedly contrasted by their chromatographic behaviors, K_m values for $(ADP\text{-}ribose)_n$, and sensitivities to monovalent salts and nucleotides. As for the molecular sizes estimated by SDS–polyacrylamide gel, poly(ADP-ribose) glycohydrolase II (M_r = 59 500) obtained here was distinct from poly(ADP-ribose) glycohydrolase I(M_r = 75 500) (Tanuma et al., 1986a). These differences in catalytic and molecular properties seem to be suitable parameters for identification of the two forms. Several poly(ADP-ribose) glycohydrolase activities have been partially and extensively purified from other sources including calf thymus (Miwa et al., 1974; Hatakeyama et al., 1986; Thomassin et al., 1990) and pig thymus (Tovassoli et al., 1983). In all cases, the glycohydrolases were from postnuclear fractions. Thus, as judged by their subcellular localization and properties, these activities appear to be classified as a poly(ADP-ribose) glycohydrolase II.

In order to compare amino acid compositions between glycohydrolases II and I, we have extensively purified both enzymes by reverse-phase HPLC on a phenyl-5PWRP column. The amino acid composition of glycohydrolase II was different, especially in Lys and Arg, from that of glycohydrolase I. The lower contents of the basic amino acid residues of glycohydrolase II may cause the low affinity for negatively charged $(ADP\text{-}ribose)_n$ and single-stranded DNA. Peptide mapping of the two forms gave rise to several discernible and distinguishable peptide bands. These results suggest that they are structurally distinct polypeptides. We conclude, therefore, that at least two subcellular forms exist, which are physically distinct, in guinea pig liver. However, we cannot rule out the possibility that these differences between the two forms may reflect modification and/or proteolysis during the purification processes. Whether these two forms result from modification, proteolysis, or different gene products will require additional investigation at the protein or genetic level. The partial polypeptide sequences and gene cloning of glycohydrolases I and II are currently under investigation.

By making the assumption that the 59 500 band on SDS gels corresponds to poly(ADP-ribose) glycohydrolase II and by estimating about 90% purity of the protein in the final preparation, the total mass of glycohydrolase II in 500 g of guinea pig liver is able to be estimated by using Table I. The result is $0.114 \times 0.90 \times (0.15^{-1}) = 0.72$ mg. Since 500 g of guinea pig liver should contain approximately 7×10^{11} cells, the number of cytoplasmic glycohydrolase II molecules/cell is calculated to be about 10 000. The number is one-fifth that of nuclear glycohydrolase I (50 000 molecules/nucleus) (Tanuma et al., 1986a). Recently, we found that in HeLa S3 cells, the two distinct subcellular forms of poly(ADP-ribose) glycohydrolase (I and II) are present in equal amount (Tanuma et al., 1986b) and that the cell cycle dependencies of their activities are different (Tanuma & Otsuka, 1991). Moreover, the two forms of poly(ADP-ribose) glycohydrolase present in human lymphocytes appear to be separately regulated during differentiation.[2] Taken together, these results clearly demonstrate that eukaryotic cells contain two distinct subcellular forms of poly(ADP-ribose) glycohydrolase.

The presence of at least two forms of poly(ADP-ribose) glycohydrolase exhibiting differences in properties and subcellular localization may be related to diversity of function for the glycohydrolases. The nuclear glycohydrolase I is probably involved in chromatin-associated events via de-poly(ADP-ribosylation) of histones and non-histone chromosomal proteins. On the other hand, the cytoplasmic glycohydrolase II may play an important role on extranuclear de-poly(ADP-ribosylation), such as in mitochondria (Kun et al., 1975; Hilz et al., 1984) and ribosomes (Thomassin et al., 1985). However, we have obtained no clear evidence that would enable us to interpret the physiological significance of the multiple forms of poly(ADP-ribose) glycohydrolase. Purification of the multiple forms of poly(ADP-ribose) glycohydrolase is a significant step in the search to define the role of the glycohydrolases in cell growth and differentiation and also paves the way for studies designed to elucidate the biological function of the metabolism of $(ADP\text{-}ribose)_n$.

[2] S. Tanuma et al., unpublished observations.

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Kinetic Comparison of Reduction and Intramolecular Electron Transfer in Milk Xanthine Oxidase and Chicken Liver Xanthine Dehydrogenase by Laser Flash Photolysis†

Mark C. Walker,‡ James T. Hazzard, and Gordon Tollin*
Department of Biochemistry, University of Arizona, Tucson, Arizona 85721
Dale E. Edmondson
Department of Biochemistry, School of Medicine, Emory University, Atlanta, Georgia 30322
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ABSTRACT: A comparative study using laser flash photolysis of the kinetics of reduction and intramolecular electron transfer among the redox centers of chicken liver xanthine dehydrogenase and of bovine milk xanthine oxidase is described. The photogenerated reductant, 5-deazariboflavin semiquinone, reacts with the dehydrogenase (presumably at the Mo center) in a second-order manner, with a rate constant ($k = 6 \times 10^7$ M^{-1} s^{-1}) similar to that observed with the oxidase [$k = 3 \times 10^7$ M^{-1} s^{-1}; Bhattacharyya et al. (1983) *Biochemistry 22*, 5270–5279]. In the case of the dehydrogenase, neutral FAD radical formation is found to occur by intramolecular electron transfer ($k_{obs} = 1600$ s^{-1}), presumably from the Mo center, whereas with the oxidase the flavin radical forms via a bimolecular process involving direct reduction by the deazaflavin semiquinone ($k = 2 \times 10^8$ M^{-1} s^{-1}). Biphasic rates of Fe/S center reduction are observed with both enzymes, which are due to intramolecular electron transfer ($k_{obs} \approx 100$ s^{-1} and $k_{obs} = 8$–11 s^{-1}). Intramolecular oxidation of the FAD radical in each enzyme occurs with a rate constant comparable to that of the rapid phase of Fe/S center reduction. The methylviologen radical, generated by the reaction of the oxidized viologen with 5-deazariboflavin semiquinone, reacts with both the dehydrogenase and the oxidase in a second-order manner ($k = 7 \times 10^5$ M^{-1} s^{-1} and 4×10^6 M^{-1} s^{-1}, respectively). Alkylation of the FAD centers results in substantial alterations in the kinetics of the reaction of the viologen radical with the oxidase but not with the dehydrogenase. These results suggest that the viologen radical reacts directly with the FAD center in the oxidase but not in the dehydrogenase, as is the case with the deazaflavin radical. The data support the conclusion that the environments of the FAD centers differ in the two enzymes, which is in accord with other studies addressing this problem from a different perspective [Massey et al. (1989) *J. Biol. Chem. 264*, 10567–10573]. In contrast, the rate constants for intramolecular electron transfer among the Mo, FAD, and Fe/S centers in the two enzymes (where they can be determined) are quite similar.

Xanthine oxidase (XO)[1] and xanthine dehydrogenase (XDH) are complex metalloflavoproteins that catalyze the oxidation of xanthine to uric acid and are thought to differ mainly in their electron acceptor specificities (O_2 and NAD^+, respectively). Since the dehydrogenase-to-oxidase interconversion has been demonstrated in enzyme preparations from a number of mammalian sources (Waud & Rajagopalan, 1976; Nakamura & Yamazaki, 1982), it is of interest to compare the properties of both forms in order to provide insights into the structural basis for the alteration in the oxidizing substrate specificity. The current evidence suggests that structural changes around the FAD site are responsible and that differences in the regions containing the Mo and the two Fe_2/S_2 centers are either nonexistent or too small to be detected by spectroscopic approaches (Barber et al., 1980). Recent comparative studies of the FAD environments in bovine XO and in chicken XDH (Massey et al., 1989) have indicated the presence of a negative charge in the flavin-binding site of the dehydrogenase but not the oxidase. Similar conclusions have been reached in studies of rat liver XDH and XO (Saito et al., 1989).

Since the suggestion by Olson et al. (1974) that electron distribution among the Mo, FAD, and the Fe/S I and Fe/S II centers of XO is kinetically rapid relative to catalytic turnover and is in accord with the relative redox potentials of the above centers, a number of experimental approaches have been employed to measure the rates of intramolecular electron

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* Corresponding author.
‡ Present address: Monsanto Agricultural Co., 800 N. Lindbergh Blvd., St. Louis, MO 63167.

[1] Abbreviations: XO, bovine milk xanthine oxidase; XDH, chicken liver xanthine dehydrogenase; dRf•, 5-deazariboflavin semiquinone, $MV^{•+}$, methylviologen cation radical.

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