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Activity of Four Allelic Forms of Glutathione S-Transferase hGSTP1-1 for Diol Epoxides of Polycyclic Aromatic Hydrocarbons

Xun Hu,* Hong Xia,* Sanjay K. Srivastava,* Christian Herzog,† Yogesh C. Awasthi,‡ Xinhua Ji,§ Piotr Zimniak,† and Shivendra V. Singh*,[1]

**Cancer Research Laboratory, Mercy Cancer Institute, Mercy Hospital of Pittsburgh, Pittsburgh, Pennsylvania 15219; †Department of Medicine and Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, and McClellan VA Hospital Medical Research, Little Rock, Arkansas 72205; ‡Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77555; and §ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702*

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Allelic forms of hGSTP1-1 which differ from each other by their catalytic properties and, structurally, by the amino acid(s) in position(s) 104 or (and) 113 are known to exist in human populations. The four possible isoforms of hGSTP1-1 with isoleucine or valine in position 104 and with alanine or valine in position 113 were produced by site-directed mutagenesis of the cDNA followed by bacterial expression and purification of the proteins. Glutathione-conjugating activity was measured with the diol epoxides of benzo(a)pyrene and chrysene, as well as with the model substrate 1-chloro-2,4-dinitrobenzene. Isoenzymes with valine in position 104 were more effective with the diol epoxides of polycyclic aromatic hydrocarbons but less effective with 1-chloro-2,4-dinitrobenzene than the isoforms with isoleucine 104. In addition, the transition A113V in the presence of V104 caused a pronounced increase in catalytic efficiency for the benzo(a)pyrene but not the chrysene diol epoxide. It is proposed that amino acid 113 functions as part of a clamp that lines the mouth of the water channel leading to the active sites of the hGSTP1-1 dimer and controls the access to substrates. Therefore, the hydrophobicity and the size of residue 113 are important in co-determining the substrate specificity of the isoenzymes. The widely different activities of the allelic isoforms toward carcinogenic diol epoxides of polycyclic aromatic hydrocarbons may help to explain the correlation between cancer susceptibility and genotype at the *hGSTP1* locus that has been found by others. © 1997 Academic Press

[1] Corresponding author: Dr. Shivendra V. Singh, Cancer Research Laboratory, Mercy Cancer Institute, Mercy Hospital of Pittsburgh, 1400 Locust Street, Pittsburgh, PA 15219. Fax: (412) 232-5753. E-mail: SSINGH@MERCY.PMHS.ORG.

Glutathione S-transferase (GST) Pi is a member of one of the four major classes of mammalian cytosolic GSTs (1-3). The enzyme, termed hGSTP1-1 in humans, has been extensively studied because of its overexpression in pre-neoplastic and tumor tissues. This behavior makes hGSTP1-1 useful as an early tumor marker, but is also responsible for drug resistance of many cancers (3-5). Of no less importance is the role of hGSTP1-1 in the metabolism and detoxification of many carcinogenic xenobiotics, among them the diol epoxides of polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene (6,7) or chrysene (8). Because of this activity, hGSTP1-1 is expected to afford protection against PAHs which are ubiquitous environmental pollutants. It has been recently demonstrated that BPDE, the diol epoxide of benzo(a)pyrene, causes mutations in the *p53* gene at the same mutational hot spots that are characteristic for lung cancers (9). Since BPDE is formed by metabolic activation from benzo(a)pyrene which is abundant in cigarette smoke, this finding established the first mechanistic, rather than statistical, link between smoking and lung cancer. Thus, hGSTP1-1 appears directly relevant to the susceptibility of individuals to smoking-related lung cancer, as well as to tumors caused by exposure to certain industrial emissions.

In contrast to the mouse (10), humans are believed to have a single functional gene encoding a Pi-class GST (11). However, early cDNA and gene sequencing results suggested the presence of at least two variants of the hGSTP1-1 protein, one with an isoleucine codon in position 104 and an alanine codon in position 113 (12-14), and another with valine codons in both of the above positions (15). This, together with evidence for polymorphism in the introns of the *hGSTP1* gene (16)

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strongly indicated that allelic forms of *hGSTP1* exist in the human population. Our finding that two hGSTP1-1 forms, both with alanine in position 113 but with either isoleucine or valine in position 104, can be identified by direct protein sequencing in the tissue of a single individual (17) confirmed the notion of allelic polymorphism at the *hGSTP1* locus, and added another variant to the two previously known. The existence of the three allelic forms, hGSTP1(I104,A113), hGSTP1(V104,A113), and hGSTP1(V104,V113), has been subsequently confirmed by analyses of genomic DNA and/or mRNA (18,19). The hGSTP1(I104,A113) allele is most frequent (18,19); interestingly, the fourth possible form, hGSTP1(I104,V113), has not been identified in the human population so far.

In light of the conservative isoleucine to valine substitution, we were surprised to find that the hGSTP1 (I104,A113) and hGSTP1(V104,A113) forms of the enzyme differed significantly from each other, both in their catalytic properties for a number of substrates and in their thermal stability (20). This result has been recently confirmed by others (19). Our previous finding that hGSTP1(I104,A113) is significantly more active than hGSTP1(V104,A113) toward 1-chloro-2,4-dinitrobenzene (CDNB) but that the ranking of the enzymes is reversed in the case of other substrates, e.g. ethacrynic acid (20), prompted us to compare the activity of the enzymes for the toxicologically important PAH diol epoxides. We have demonstrated that hGSTP1 (V104,A113) is more efficient than hGSTP1(I104,A113) in the glutathione conjugation of *anti*-BPDE (7) as well as the diol epoxide of chrysene, CDE (8), and provided a structure-based rationalization of these findings. In the present communication, we report for the first time the kinetic properties of all four hGSTP1 variants, including hGSTP1(I104,V113), with CDNB, BPDE, and CDE as substrates.

MATERIALS AND METHODS

Materials. GSH and epoxy-activated Sepharose 6B were purchased from Sigma (St. Louis, MO). (±)-*Anti*-CDE and (+)-*anti*-BPDE were procured from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Chemsyn Science Laboratories, Lenexa, KS).

Site directed mutagenesis, and expression and purification of hGSTP1-1 isoforms. Expression vectors pET9a carrying the cDNAs for hGSTP1(I104,A113) and hGSTP1(V104,A113) have been prepared by us previously (20), and were used for the expression of the corresponding proteins. In addition, both plasmids served as the template for site-directed mutagenesis (Morph kit, 5 Prime - 3 Prime Inc., Boulder, CO), using the antisense mutagenic oligonucleotide 5′-TCCTTGCCCaCCTCATAG (the mutation site is shown in lower case) to change the A113 codon GCG to GTG which encodes valine, and is identical to that found in the genomic sequence of hGSTP1(V113) (15). The four variants of hGSTPl-1 were purified by glutathione affinity chromatography according to (21) with slight modifications (20), were dialyzed against TKE buffer (50 mM Tris-HCl, pH 7.5, 2.5 mM KCl, 0.5 mM EDTA), and stored at −20°C until used.

Determination of GST activity toward (+)-anti-BPDE and (±)-anti-CDE. The reaction mixtures (in a final volume of 0.1 ml) contained: TKE buffer, 2 mM GSH, the desired concentration of (+)-*anti*-BPDE or (±)-*anti*-CDE, and 30 μg/ml of the hGSTP1-1 protein. The reaction was initiated by adding the diol epoxide, and the reaction mixture was incubated for 30 s at 37°C. The reaction was terminated by rapid mixing with 0.1 ml of cold acetone. Unreacted substrate was removed by extraction with ethyl acetate saturated with TKE buffer, and the glutathione conjugates in the aqueous phase were quantitated by reverse-phase HPLC as described by us previously (22,23). In preliminary experiments, the reaction was optimized by varying both protein concentration and incubation time. A typical reaction is illustrated in Fig. 1. The kinetics of glutathione conjugation of both PAH diol epoxides adhered to the Michaelis-Menten model (Fig. 2); the kinetic constants were estimated by nonlinear regression fitting of a hyperbolic function to the data points using the computer program Kinet.

Determination of GST activity toward CDNB. The assay was carried out at 25°C according to (24), except that CDNB concentrations were varied between 0.1 and 5.0 mM in the presence of 2.5 mM GSH. The enzyme concentrations were 0.05 μg/ml and 0.1 μg/ml for hGSTP1-1(I104,V113) and hGSTP1-1(V104,V113), respectively. Michaelis-Menten kinetic parameters were calculated as described above.

RESULTS AND DISCUSSION

Allelic variants of hGSTP1-1 have been identified that have either isoleucine or valine in position 104, and either alanine or valine in position 113. Of the four possible combinations of these variants, three (hGSTP1-1 (I104,A113), hGSTP1-1 (V104,A113), and hGSTP1-1(V104,V113)) have been found in human populations (17-19). These allelic forms will be denoted I-A, V-A, and V-V for the purpose of further discussion. The fourth form, hGSTP1-1(I104,V113) or I-V in shorthand, may be rare, or it may be absent from the gene pool. Since no a priori reason for its absence is evident, we included all four forms in the present study of the metabolism of diol epoxides of PAHs by hGSTP1-1.

The catalytic efficiency of four hGSTP1-1 isoforms for the model substrate CDNB and for two PAH diol epoxides, (+)-*anti*-BPDE and (±)-*anti*-CDE, is shown in Fig. 3. In the case of CDNB, high catalytic efficiency required the presence of Ile in position 104, regardless of whether position 113 was occupied by Ala or Val. This finding confirms and extends our previous observations regarding amino acid 104 (20), and is consistent with our interpretation that the size of the hydrophobic side chain of residue 104 influences the number and position of conserved water molecules in the active site, and thus affects the binding of substrate and/or reaction intermediate (7). Interestingly, the high k_{cat}/K_M of the I-A enzyme is due to a low K_M (Fig. 4A), while in the case of I-V, a k_{cat} that is significantly higher than that of the other isoforms (Fig. 5A) is mainly responsible for the increased catalytic efficiency. A mechanistic interpretation of this result will require further studies. It is also noteworthy that the K_M of form V-V for CDNB, 1.3 ± 0.4 mM (Fig. 4A), is significantly lower

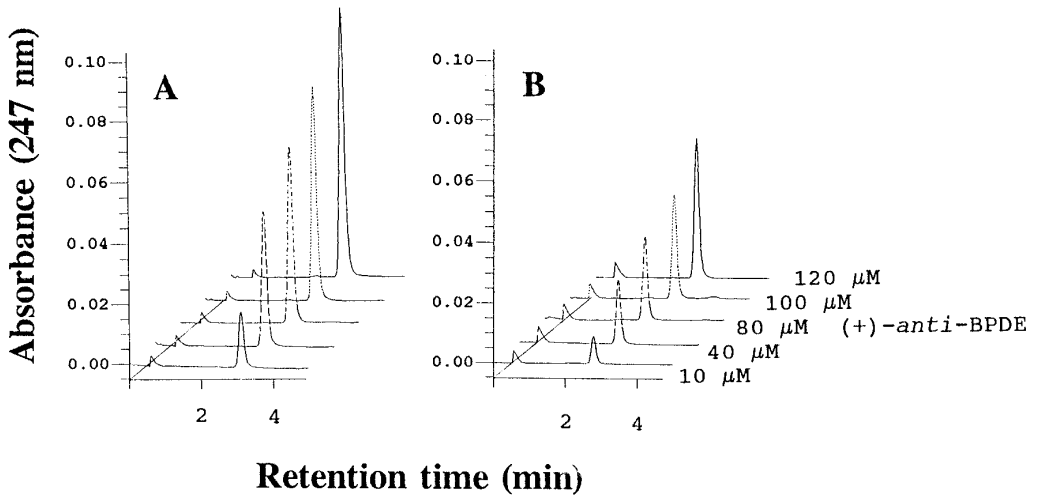

FIG. 1. Reverse-phase HPLC analysis of water-soluble products resulting from the reaction of 2 mM glutathione and different concentrations of (+)-*anti*-BPDE in the presence of 30 μg/ml of hGSTP1-1(V104,V113) (A), and the same concentration of hGSTP1-1(I104,V113) (B).

than the value of 3.1 ± 0.2 mM that was recently reported (19). We believe this discrepancy to be due to different experimental conditions. In our hands, the amount of protein used per assay in (19), 0.015 units or 0.3 μg, leads to a loss of linearity of absorbance change over time within the 2 min assay, and thus introduces errors into the reported kinetic parameter estimates (19).

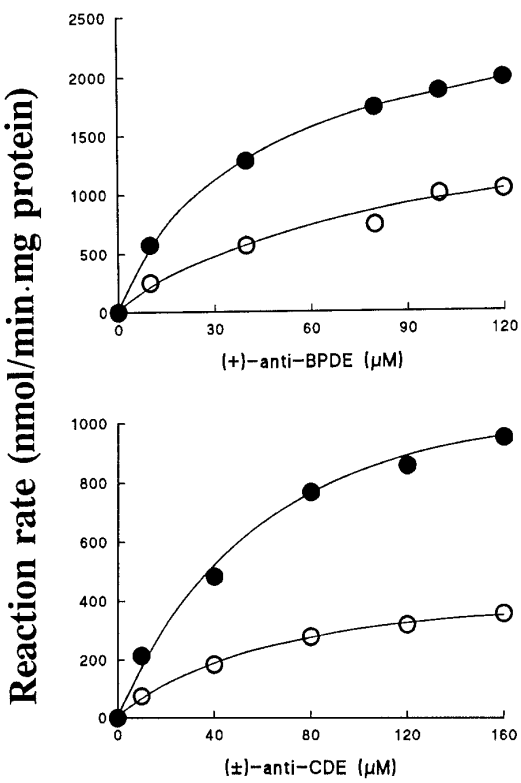

FIG. 2. The rate of glutathione conjugation of (+)-*anti*-BPDE or (±)-*anti*-CDE as a function of diol epoxide concentration in the presence of 30 μg/ml of hGSTP1-1(V104,V113) (●), and the same concentration of hGSTP1-1(I104,V113) (○). The glutathione conjugates of the diol epoxides were quantitated by HPLC as described in "Materials and Methods."

As we already reported (7,8), the turnover number of isoform V-A with both (+)-*anti*-BPDE and (±)-*anti*-CDE is significantly higher that that of isoform I-A (Fig. 5B and C). The higher activity of the V104 versus I104 form also holds for the V-V / I-V pair of enzymes, albeit to a lesser degree (Fig. 5B and C). Except for the V-A enzyme assayed with (+)-*anti*-BPDE where a high K_M (Fig. 4B) cancels the high activity of that enzyme when the catalytic efficiency is calculated, in the remaining cases the increase in activity upon the I104V transition is also reflected in an increased catalytic efficiency for PAH diol epoxides (Fig. 3B, C). These data indicate that a I104V transition, with position 113 held invariant, leads to an increase of activity for PAH diol epoxides. This generalization is in agreement with our structural interpretation proposed in (7) according to which residue 104 determines the arrangement of active site water molecules. This, in turn, stabilizes (for V104) or destabilizes (for I104) the binding of PAH diol epoxide substrates.

The role of residue 113 can be examined by considering the transition from Ala-113 to Val while holding amino acid 104 invariant. In the case of the suboptimal I104, the A113V transition had little or no effect (panels B and C in Figs. 3-5). In the V104 context, the same A113V transition had no effect on the K_M for (±)-*anti*-CDE, and a very slight negative effect on the k_{cat} for this substrate (Figs. 4 and 5, panel C). The situation was strikingly different with (+)-*anti*-BPDE as substrate. Here, the A113V transition in the V104 background led to a two-fold drop in k_{cat} that was, however,

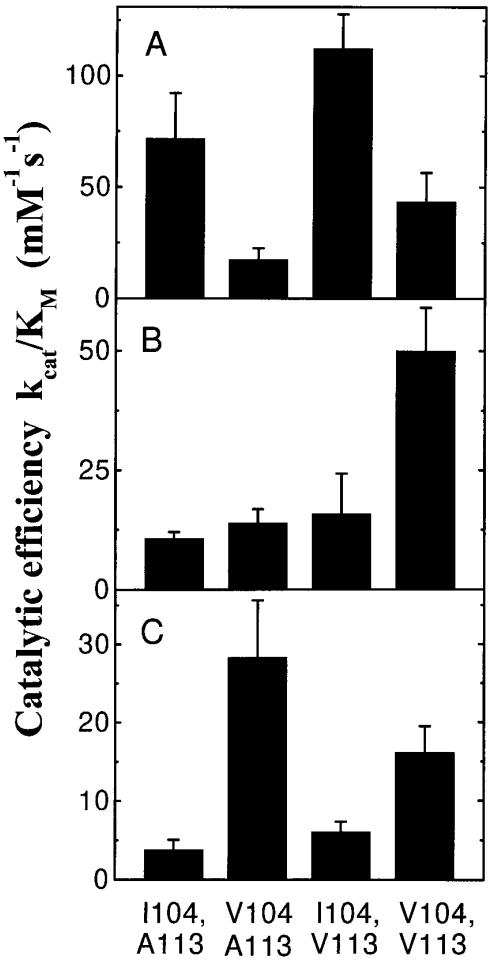

FIG. 3. Catalytic efficiencies of hGSTP1-1(I104,A113), hGSTP1-1(V104,A113), hGSTP1-1(I104,V113), and hGSTP1-1(V104,V113) with CDNB (A), (+)-*anti*-BPDE (B), and (±)-*anti*-CDE (C). The values were calculated from individual kinetic parameters (Figs. 4 and 5). The standard deviation values (bars) were estimated according to (26, page 91).

overcompensated by a sharp decrease in K_M, resulting in a catalytic efficiency of the V-V form for (+)-*anti*-BPDE that is 3 to 5 times that of the other isozymes (Fig. 3B).

Residue 104 of hGSTP1-1 is an integral part of the active site of the protein (7,20,25), and its role in the function of the enzyme can be interpreted in terms of a direct effect on the active site structure (7,25). In contrast, residue 113, which is located at the tip of the helix-turn-helix motif between helices 4 and 5 (Fig. 6A), is far removed from the active site. The distance between carbon atom CB of amino acid 113 and the sulfur atom of active site-bound glutathione is 21.6 Å within the same subunit, and as much as 26.8 Å across the subunit interface (25). Therefore, the impact of the A113V transition on activity that was outlined above cannot be attributed to direct active site modification.

As illustrated in Fig. 6, the helix-turn-helix motifs of the two subunits of dimeric GSTs are positioned at the opening of the solvent channel that leads to both active sites. We postulate that a xenobiotic substrate molecule may initially interact with the helix-turn-helix motifs, and be only subsequently transferred to the active center. A close-up view of a model of hGSTP1-1(V104,V113) complexed with a glutathione conjugate of phenanthrene 9,10-oxide [(9R,10R)-GSPhen] was constructed according to the crystal structure (25), and is shown in Fig. 6B. The residue next to V113, and also facing the solvent channel, is G114. These two amino acids, and the corresponding residues of the second subunit, face each other across the solvent channel, forming a "clamp" with hydrophobic tips. The A113V transition would increase the hydrophobicity of the clamp, and may in addition decrease the distance between the two tips, or jaws, of the clamp, thus facilitat-

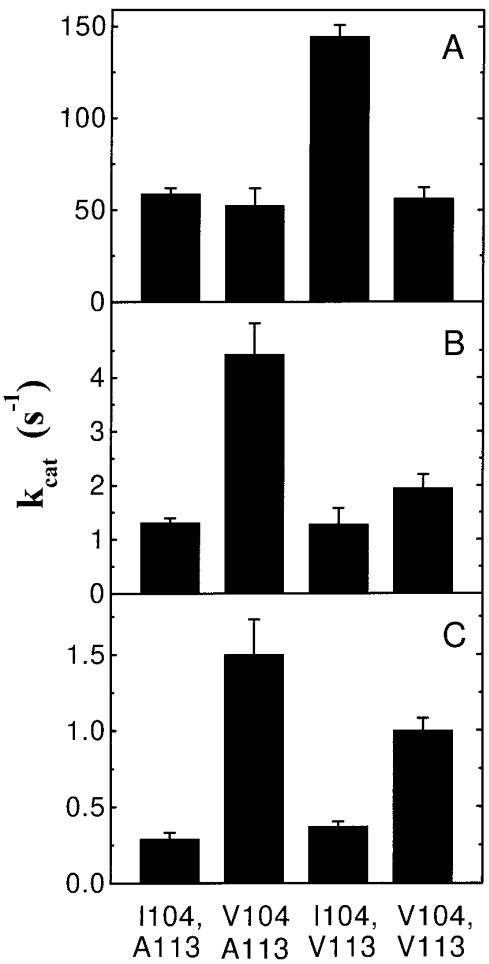

FIG. 4. Turnover numbers of hGSTP1-1(I104,A113), hGSTP1-1(V104,A113), hGSTP1-1(I104,V113), and hGSTP1-1(V104,V113) with CDNB (A), (+)-*anti*-BPDE (B), and (±)-*anti*-CDE (C). Data for (+)-*anti*-BPDE with hGSTP1-1(I104,A113) and hGSTP1-1(V104,A113) are from (7), and those for (±)-*anti*-CDE and CDNB with the same two enzymes are from (8) and (20), respectively.

ing the interaction of large and hydrophobic molecules with the clamp.

It can be predicted that the interaction of substrate molecules with the clamp would have two effects. First, the local concentration of substrate at the mouth of the solvent channel and thus in the vicinity of the active site would increase, which should lower the apparent K_M of the enzyme for this substrate. Second, the cost of the above gain would be a competition between the clamp and the active site for the substrate, potentially leading to a decrease in k_{cat}. It can be further predicted that the effects outlined above will be most pronounced with bulky substrates which can tightly interact with the clamp, but will be weaker or absent with smaller or hydrophilic substrates. Finally, the concentrative effect of the clamp would become irrelevant if the substrate is poorly metabolized in the active site. The above predictions are borne out by the results presented in this communication. The hydrophobic clamp formed by V113 residues is more effective with the bulkier BPDE than with the smaller CDE molecule, it functions only in conjunction with V104 in the active site, causes a slight decrease of the k_{cat}, and, in the optimal situation, leads to a sharp decrease in the K_M.

In summary, the differences in catalytic properties of the four variants of hGSTP1-1 can be rationalized in terms of their structure. No less important are the physiological implications of these differences. Three of the four possible allelic forms have been found in the human population (18,19), and their frequency differed between healthy controls and cancer patients (18), implying that the disparate activities, presumably toward environmental carcinogens, are relevant in co-determining overall susceptibility to certain cancers. Thus, the results presented in this communication contribute to a molecular understanding of the role of hGSTP1-1 in cancer etiology, and may be applied as a tool in risk prediction.

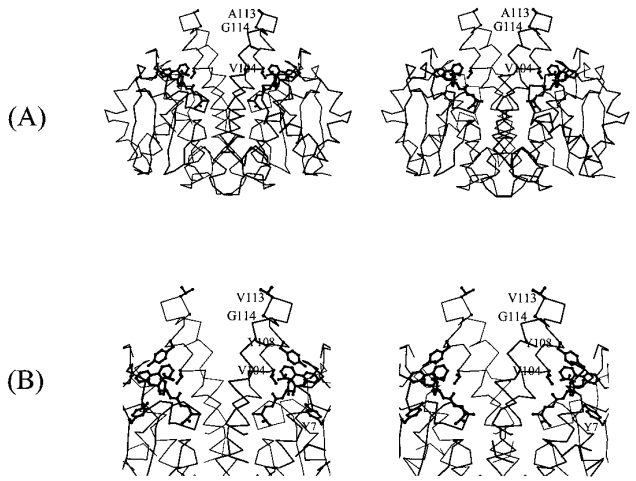

FIG. 6. Panel A. Stereo view of the Cα trace of the hGSTP1-1(V104,A113) complexed with (9R,10R)-GSPhen (the glutathione conjugate of 9R,19S-phenanthrene), viewed perpendicular to the pseudo 2-fold axis. The side chains of V104, A113, and the product molecule (9R,10R)-GSPhen are illustrated as ball-and-stick models. Panel B. Close-up stereo view of the Cα trace of a model of the hGSTP1–1(V104,V113)●(9R,10R)-GSPhen complex shown in the same orientation as in panel A. In addition to V104, A113, and the product molecule, the side chains of Y7 and Y108 are also highlighted in panel B. For clarity, residues are labeled in one subunit only.

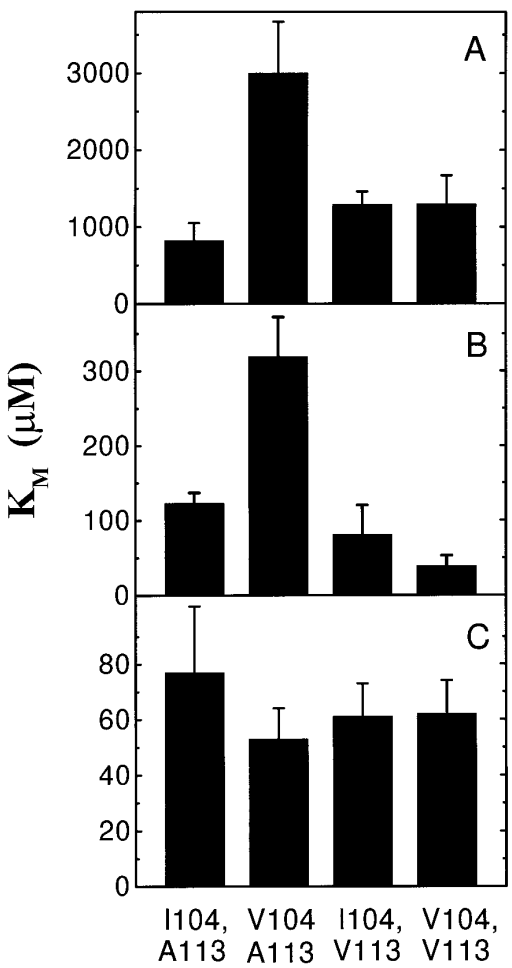

FIG. 5. Michaelis constants of hGSTP1-1(I104,A113), hGSTP1-1(V104,A113), hGSTP1-1(I104,V113), and hGSTP1-1(V104,V113) with CDNB (A), (+)-*anti*-BPDE (B), and (±)-*anti*-CDE (C). Data for (+)-*anti*-BPDE with hGSTP1-1(I104,A113) and hGSTP1-1(V104,A113) are from (7), and those for (±)-*anti*-CDE and CDNB with the same two enzymes are from (8) and (20), respectively.

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