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Sulfonation of environmental estrogens by zebrafish cytosolic sulfotransferases☆

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Abstract

Environmental estrogen-like chemicals are increasingly recognized as a potential hazardous factor for wildlife as well as humans. We have recently embarked on developing a zebrafish model for investigating the role of sulfonation in the metabolism and adverse functioning of environmental estrogens. Here, we report on a systematic investigation of the sulfonation of representative environmental estrogens (bisphenol A, 4-*n*-octylphenol, 4-*n*-nolylphenol, diethylstilbestrol, and 17α-ethynylestradiol) by zebrafish cytosolic sulfotransferases (STs). Of the seven enzymes tested, four zebrafish STs (designated ZF ST #2, ZF ST #3, ZF ST #4, and ZF DHEA ST) exhibited differential sulfonating activities toward the five environmental estrogens tested, with ZF ST #3 being more highly active than the other three. It was further demonstrated that bisphenol A, 4-*n*-octylphenol, and 4-*n*-nonylphenol exerted concentration-dependent inhibition of the sulfonation of 17β-estradiol, implying a potential role of these environmental estrogens in interfering with the sulfonation, and possibly homeostasis, of endogenous estrogens. Kinetic studies revealed that the mechanism underlying the inhibition by bisphenol A or 4-*n*-nonylphenol to be of the competitive type.

Sulfotransferases (STs) are enzymes, present in both plants and animals, that catalyze the sulfonation of a variety of compounds containing hydroxyl or amino groups, using 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as the sulfonyl group donor [1–3]. While the membrane-bound STs use proteins, glycolipids, and other macromolecules as substrates, the cytosolic STs sulfate low-molecular weight endogenous and xenobiotic compounds. This serves to both regulate the levels and activities of endogenous molecules such as thyroid/steroid hormones and catecholamine hormones/neurotransmitters, as well as detoxify dietary, therapeutic, and environmental xenobiotics [4,5].

In recent years, environmental estrogen-like chemicals have been increasingly recognized as a potential hazardous factor for wildlife as well as humans [6]. In general, they are able to bind to estrogen receptors and thereby mimic estrogenic actions [7] or interfere with the action of enzymes which help regulate the level of endogenous estrogens and other hormones [8]. Some examples of this diverse group of compounds are diethylstilbestrol, bisphenol A, nonylphenol, polychlorinated biphenyls, and dichlorodiphenyltrichloroethane [9]. These environmental estrogens are becoming ubiquitous in the environmental and making their way into the food chain. Among other adverse effects, the environmental estrogens have been implicated in the abnormal sexual development of reptiles and bird [10,11], the decline in sperm quality of men [12,13], and an increased incidence of human breast cancer [14,15]. We have recently demonstrated the sulfonation of some environmental estrogens by human cytosolic STs [16,17]. The role of sulfonation in the metabolism and

☆ *Abbreviations:* ST, sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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modulation of environmental estrogens in the context of physiology, however, remains unknown. To obtain such information, a suitable animal model is required.

Zebrafish has in recent years emerged as a popular animal model for a wide range of studies [18,19]. Its advantages, compared with mouse, rat, or other vertebrate animal models, include the small size, availability of relatively large number of eggs, rapid development externally of virtually transparent embryo, and short generation time. These unique characteristics of the zebrafish make it an excellent model for a systematic investigation on the physiological involvement of cytosolic STs, including the sulfonation of environmental estrogens. A prerequisite for using zebrafish in these studies, however, is the identification of the various cytosolic STs and their functional characterization. We have recently embarked on the molecular cloning of zebrafish cytosolic STs, and have expressed and purified a number of these enzymes [20–22].

In this communication, we report on the characterization of the sulfonating activities of zebrafish cytosolic STs toward representative environmental estrogens. Kinetic parameters of the sulfonation of these environmental estrogens were determined. Moreover, the inhibitory effects of these compounds on the sulfonation of 17β-estradiol, an endogenous estrogen, and the underlying mechanism were studied.

Materials and methods

Materials. Bisphenol A, diethylstilbestrol, 4-*n*-octylphenol, 4-*n*-nonylphenol, 17α-ethynylestradiol, 17β-estradiol, dehydroepiandrosterone (DHEA), estrone, aprotinin, thrombin, adenosine 5′-triphosphate (ATP), PAPS, sodium dodecyl sulfate (SDS), *N*-2-hydroxylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), Trizma base, isopropyl β-D-thiogalactopyranoside (IPTG), inorganic pyrophosphatase, and dithiothreitol (DTT) were obtained from Sigma Chemical. Human ATP sulfurylase/APS kinase was prepared as described previously [23]. BL21 (DE3) *Escherichia coli* host strain was from Novagen. Carrier free sodium [^{35}S]sulfate was from ICN Biomedicals. Cellulose thin-layer chromatography (TLC) plates were from EM Science. All other reagents were of the highest grade commercially available.

Expression and purification of recombinant zebrafish cytosolic STs. We have previously cloned seven zebrafish cytosolic ST cDNAs, packaged them individually in pGEX-2TK or pET23c prokaryotic expression vector, and transformed the plasmid constructs into BL21 (DE3) cells [20–22]. Transformed BL21 (DE3) cells were grown in 1 L of LB broth containing 50 μg/mL ampicillin. After the cell density reached 0.7 OD_{600nm}, 0.1 mM (for pGEX-2TK constructs) or 0.4 mM (for pET23c constructs) IPTG was added to induce the production of recombinant zebrafish cytosolic ST. After a 5-h induction at 37 °C, the cells were collected by centrifugation and homogenized in 20 mL of a lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA) using an Aminco French Press. Recombinant zebrafish cytosolic STs present in the cell homogenates were purified using previously established procedures [20–22], analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), determined for protein concentration, and used in the enzymatic assays.

Enzymatic assay. The ST assays were performed using PAP[^{35}S] as the sulfonyl group donor. The standard assay reaction mixture contained, in a final volume of 25 μL, 50 mM Hepes, pH 7.0, 14 μM PAP[^{35}S], and 50 μM substrate. The reaction was started by the addition of enzyme (0.025 μg), allowed to proceed for 3 min at 28 °C, and stopped by heating at 100 °C for 2 min. Upon brief centrifugation to pellet down the precipitates, 1 μL aliquot of the reaction mixture was spotted on a cellulose plate for the TLC analysis of [^{35}S]sulfated product based on our previously established procedure [24]. For the kinetic studies on the sulfonation of endogenous estrogens (estrone and 17β-estradiol) and environmental estrogens, varying concentrations (ranging from 0.5 to 50 μM) of these substrate compounds and 50 mM Hepes at pH 7.0 were used. To determine the inhibitory effects of bisphenol A, 4-*n*-octylphenol, and 4-*n*-nonylphenol, enzymatic assays in the presence of varying concentrations (ranging from 0 to 1 mM) of these environmental estrogens were performed with 50 μM 17β-estradiol as substrate. To investigate the mechanism underlying the inhibition of the sulfonation of 17β-estradiol by bisphenol A or 4-*n*-nonylphenol, enzymatic assays using varying concentrations (5–20 μM) of 17β-estradiol in the presence of fixed concentrations of bisphenol A (0, 400, and 800 μM) or 4-*n*-nonylphenol (0, 20, and 50 μM) were performed.

Miscellaneous methods. PAP[^{35}S] (carrier-free) was synthesized from ATP and carrier-free [^{35}S]sulfate using the human bifunctional ATP sulfurylase/APS kinase as described previously [23]. The PAP[^{35}S] synthesized was then adjusted to the desired concentration and specific activity by the addition of cold PAPS. SDS–PAGE was performed on a 12% polyacrylamide gel using the method of Laemmli [25]. Protein determination was based on the method of Bradford with bovine serum albumin as the standard [26].

Results and discussion

In view of the mounting evidence of the hazardous effects of environmental estrogens on both wildlife and humans [6,10,11], we became interested in investigating whether sulfonation, a Phase II detoxification pathway, might be involved in the metabolism and modulation of these compounds. In our earlier studies [16,17], we demonstrated indeed the occurrence of the sulfonation of representative environmental estrogens by human cytosolic STs. We recently embarked on developing the zebrafish as an animal model for investigating the sulfonation of environmental estrogens in the context of physiology. We started by cloning the various cytosolic STs present in zebrafish [20–22]. The seven distinct zebrafish cytosolic STs cloned to date were bacterially expressed and examined with respect to their environmental estrogen-sulfonating activities, as well as the inhibitory effects of environmental estrogens on the sulfonation of an endogenous estrogen, 17β-estradiol.

Preparation and characterization of zebrafish cytosolic STs with environmental estrogens as substrates

The seven zebrafish cytosolic STs previously cloned [20–22] were bacterially expressed and purified. Fig. 1 shows the SDS–gel electrophoretic pattern of the purified zebrafish cytosolic STs. These purified STs were first assayed for sulfonating activity towards some typical environmental estrogens (including bisphenol A, 4-*n*-oc-

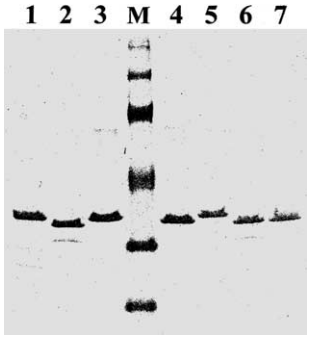

Fig. 1. SDS–gel electrophoretic patterns of purified zebrafish cytosolic STs. Samples analyzed in lanes 1–7 were: ZF ST #1, ZF ST #2, ZF ST #3, ZF ST #4, ZF ST #5, ZF DHEA ST, and ZF NST. Protein molecular weight markers co-electrophoresed in lane M are β-lactoglobulin ($M_r = 18,400$), carbonic anhydrase ($M_r = 29,000$), ovalbumin ($M_r = 43,000$), bovine serum albumin ($M_r = 68,000$), phosphorylase b ($M_r = 97,400$), and myosin (H-chain) ($M_r = 200,000$).

tylphenol, 4-*n*-nonylphenol, diethylstilbestrol, and 17α-ethynylestradiol) and an endogenous estrogen, 17β-estradiol. In these experiments, a saturating concentration of PAPS, i.e., 14 and 50 μM of environmental estrogens or 17β-estradiol were used in the assays. The results compiled in Table 1 showed that three of the seven zebrafish STs (ZF ST #1, ZF ST #5, and ZF NST) were inactive. The other four zebrafish STs (ZF ST #2, ZF ST #3, ZF ST #4, and ZF DHEA ST) exhibited differential sulfonating activities toward the five environmental estrogens tested, with ZF ST #3 being more highly active than the other three. We therefore decided to examine in greater detail the kinetic parameters of ZF ST #3 in catalyzing the sulfonation of these environmental estrogens and 17β-estradiol. In these experiments, varying concentrations of environmental estrogens or 17β-estradiol were used in the assays. The kinetic constants determined are compiled in Table 2. Of the five environmental estrogens tested, 4-*n*-octylphenol and diethylstilbestrol appeared to be better substrates for the enzyme, as reflected by the calculated V_{max}/K_m values.

An important issue is whether and how the presence of environmental estrogens may interfere with the sulfonation of endogenous estrogens. To resolve this issue, we first tested the inhibitory effects of environmental estrogens on the sulfonation of 17β-estradiol. Enzymatic assays were carried out using 5 μM 17β-estradiol as substrate in the presence of different concentrations (ranging from 0 to 1 mM) of bisphenol A, 4-*n*-octylphenol, or 4-*n*-nonylphenol. As shown in Fig. 2, all three environmental estrogens tested exerted concentration-dependent inhibition of the sulfonation of 17β-estradiol. The IC_{50} values determined based on the results shown in the figure were 90, 5, and 17.5 μM, respectively, for bisphenol A, 4-*n*-octylphenol, and 4-*n*-nonylphenol. These results indicated clearly the potential role of environmental estrogens in interfering with the sulfonation, and possibly homeostasis, of endogenous estrogens.

To further investigate the mechanism underlying the inhibition of 17β-estradiol sulfonation by environmental estrogens, enzymatic assays using varying concentrations (5–20 μM) of the substrate, 17β-estradiol, in the presence of fixed concentrations of bisphenol A (0, 400, and 800 μM) or 4-*n*-nonylphenol (0, 20, and 50 μM) were performed. Data obtained were used to generate the Lineweaver–Burk double-reciprocal plots. As shown in the double reciprocal plots generated (Fig. 3), the lines corresponding to the various concentrations of bisphenol A (Part A) or 4-*n*-nonylphenol (Part B) tested, while crossing the *X*-axis at different positions, appeared to converge within a narrow region on the *Y*-axis. These

Table 2
Kinetic constants of ZF ST #3 with environmental estrogens and endogenous estrogens as substrates[a]

Substrate	V_{max} (nmol/min/mg)	K_m (μM)	V_{max}/K_m
Bisphenol A	15.4	31.1	0.5
4-*n*-Octylphenol	356	18.5	19.2
4-*n*-Nonylphenol	158	27.3	5.8
Diethylstilbestrol	208	9.0	23.1
17α-Ethynylestradiol	136	16.1	8.4
Estrone	366.4	12.5	29.3
17β-Estradiol	175.4	15.1	11.6

[a] Data shown represent mean values derived from three determinations.

Table 1
Specific activities of zebrafish cytosolic STs with environmental estrogens as substrates[a]

Specific activity (nmol/min/mg)							
Substrate	ZF ST #1	ZF ST #2 (SULT1 ST#1)	ZF ST #3 (SULT1 ST #2)	ZF ST #4	ZF ST #5	ZF DHEA ST (SULT2 ST)	ZF NST
Bisphenol A	ND[a]	ND	7.6 ± 0.4	1.7 ± 0.3	ND	ND	ND
4-*n*-Octylphenol	ND	51.7 ± 5.0	68.2 ± 5.3	74.7 ± 2.4	ND	ND	ND
4-*n*-Nonylphenol	ND	7.0 ± 0.5	34.9 ± 2.6	19.4 ± 0.6	ND	0.6 ± 0.1	ND
Diethylstilbestrol	ND	0.6 ± 0.3	51.4 ± 5.8	8.6 ± 1.0	ND	ND	ND
17α-Ethynylestradiol	ND	ND	61.7 ± 1.5	2.0 ± 0.3	ND	ND	ND
17β-Estradiol	ND	ND	79.0 ± 4.0	2.4 ± 0.6	ND	0.6 ± 0.1	ND

[a] ND, activity not detected. Data shown represent means ± SD derived from three determinations.

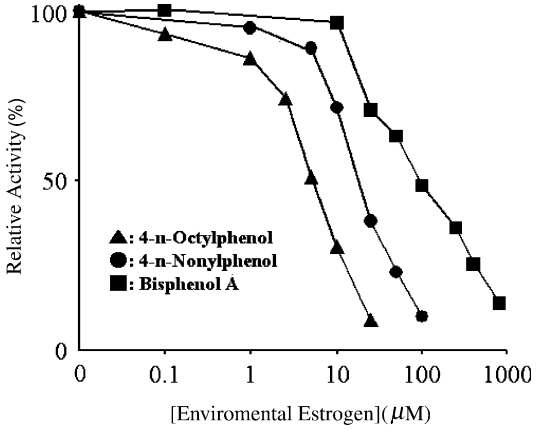

Fig. 2. Inhibition of 17β-estradiol sulfonation by 4-*n*-octylphenol (▲), 4-*n*-nonylphenol (●), and bisphenol A (■). Enzymatic assays in the presence of varying concentrations of these environmental estrogens were performed with 50 μM 17β-estradiol as substrate. Data were calculated based on the activity determined in the absence of environmental estrogen as 100%.

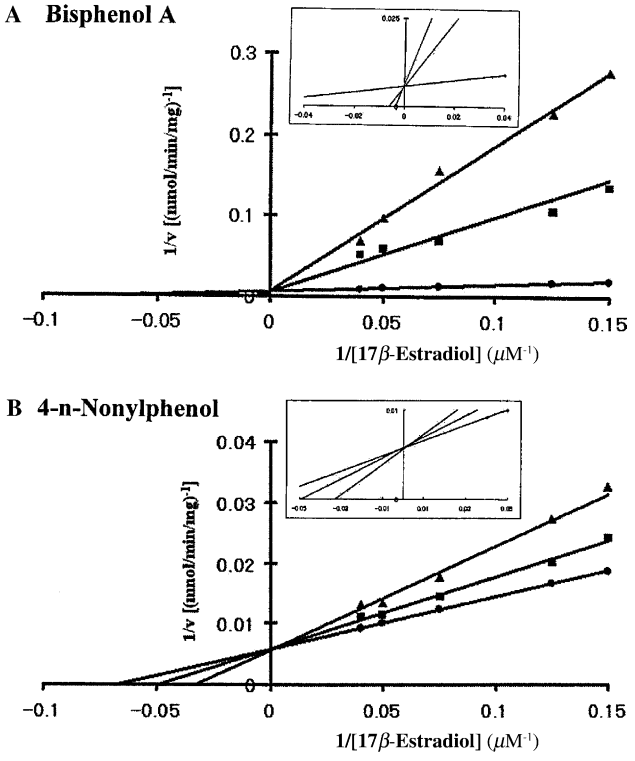

Fig. 3. (A) Lineweaver–Burk double-reciprocal plot of ZF ST #3 with 17β-estradiol as substrate in the presence of different concentrations of bisphenol A. Concentrations of bisphenol A tested were: 0 μM (●), 400 μM (■), and 800 μM (▲). (B) Lineweaver–Burk double-reciprocal plot of ZF ST #3 with 17β-estradiol as substrate in the presence of different concentrations of 4-*n*-nonylphenol. Concentrations of 4-*n*-nonylphenol tested were: 0 μM (●), 20 μM (■), and 50 μM (▲). Concentrations of 17β-estradiol are expressed in μM and velocities are expressed as nmol of product formed/min/mg enzyme. Each data point represents the mean value derived from three determinations.

Table 3
Kinetic constants of ZF ST #3 at different concentrations of bisphenol A or 4-*n*-nonylphenol with 17β-estradiol as substrate[a]

Inhibitor concentration (μM)	V_{max} (nmol/min/mg)	K_m (μM)	V_{max}/K_m
Bisphenol A			
0	175.4	15.1	11.6
400	175.4	162.1	1.1
800	158.7	286.5	0.6
4-*n*-*Nonylphenol*			
0	175.4	15.1	11.6
20	172.4	20.4	8.5
50	178.6	30.6	5.8

[a] Data shown represent mean values derived from three determinations.

results indicated that the V_{max} value of the zebrafish EST for 17β-estradiol did not change much in the presence of bisphenol A or 4-*n*-nonylphenol. Whereas the K_m increased dramatically with increasing concentrations of bisphenol A or 4-*n*-nonylphenol. K_m and V_{max}, as well as V_{max}/K_m, calculated from the Lineweaver–Burk double reciprocal plots are compiled in Table 3. These data imply likely a competitive-type of inhibition.

In conclusion, we report in this paper the sulfonation of some representative environmental estrogens by zebrafish cytosolic STs, and the inhibitory effects of these compounds on the sulfonation of 17β-estradiol, an endogenous estrogen. These studies provide a basis for further investigation into the role of sulfonation in the metabolism and adverse functioning of environmental estrogen using the zebrafish as a model.

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References

[1] G.J. Mulder, W.B. Jakoby, in: G.J. Mulder (Ed.), Conjugation Reaction in Drug Metabolism, Taylor & Francis, London, 1990, pp. 107–161.
[2] C. Falany, J.A. Roth, in: E.H. Jeffery (Ed.), Human Drug Metabolism: From Molecular Biology to Man, CRC Press, Boca Raton, FL, 1993, pp. 101–115.
[3] R. Weinshilboum, D. Otterness, in: F.C. Kaufmann (Ed.), Conjugation–Deconjugation Reaction in Drug Metabolism and Toxicity, Springer, Berlin, 1994, pp. 45–78.
[4] M.W.H. Coughtrie, S. Sharp, K. Maxwell, N.P. Innes, Chem. Biol. Interact. 109 (1998) 3–27.
[5] M.W. Duffel, in: F.P. Guengerich (Ed.), Comprehensive Toxicology, Elsevier, Oxford, 1997, pp. 365–383.
[6] D. Roy, M. Palangat, C.-W. Chen, R.D. Thomas, J. Colerangle, A. Atkinson, Z.-J. Yan, J. Toxicol. Environ. Health 50 (1997) 1–29.
[7] T.J. Ridgway, H. Wiseman, Biochem. Soc. Trans. 26 (1998) 675–680.

[8] M.H. Kester, S. Bulduk, D. Tibboel, W. Meinl, H. Glatt, C.N. Falany, M.W. Coughtrie, A. Bergman, S.H. Safe, G.G. Kuiper, A.G. Schuur, A. Brouwer, T.J. Visser, Endocrinology 141 (2000) 1897–1900.
[9] T.J. Ridgway, H. Wiseman, Biochem. Soc. Trans. 26 (1998) 675–680.
[10] L.J. Guillette Jr., T.S. Gross, D.A. Gross, A.A. Rooney, H.F. Percival, Environ. Health Prospect. (Suppl. 103) (1995) 31–36.
[11] D.M. Fry, Environ. Health Prospect. (103) (1995) 165–171.
[12] E. Carlsen, A. Giwercman, N. Keiding, N.E. Skakkebaek, Br. Med. J. 305 (1992) 609–613.
[13] J. Auger, J.M. Kunstmann, F. Czyglik, P. Jouannet, New Engl. J. Med. 332 (1995) 281–285.
[14] F. Falck Jr., A. Ricci Jr., M.S. Wolff, J. Godbold, P. Deckers, Arch. Environ. Health 47 (1992) 143–146.
[15] M.S. Wolff, P.G. Toniolo, E.W. Lee, M. Rivera, N. Dubin, J. Natl. Cancer Inst. 85 (1992) 648–652.
[16] M. Suiko, Y. Sakakibara, M.-C. Liu, Biochem. Biophys. Res. Commun. 267 (2000) 80–84.
[17] T.G. Pai, T. Sugahara, M. Suiko, Y. Sakakibara, F. Xu, M.-C. Liu, Biochim. Biophys. Acta 1573 (2002) 165–170.
[18] J.P. Briggs, Am. J. Physiol. Regul. Integr. Comp. Physiol. 282 (2002) R3–R9.
[19] A.C. Ward, G.J. Linschke, Front. Biosci. 7 (2002) d827–d833.
[20] T. Sugahara, C.C. Liu, T.G. Pai, M.-C. Liu, Biochem. Biophys. Res. Commun. 300 (2003) 725–730.
[21] T. Sugahara, C.-C. Liu, T.G. Pai, P. Collodi, M. Suiko, Y. Sakakibara, K. Nishiyama, M.-C. Liu, Eur. J. Biochem. 270 (2003) 2404–2411.
[22] T. Sugahara, C.-C. Liu, G. Carter, T.G. Pai, M.-C. Liu, Arch. Biochem. Biophys. 414 (2003) 67–73.
[23] K. Yanagisawa, Y. Sakakibara, M. Suiko, Y. Takami, T. Nakayama, H. Nakajima, K. Takayanagi, Y. Natori, M.-C. Liu, Biosci. Biotechnol. Biochem. 62 (1998) 1037–1040.
[24] M.-C. Liu, F. Lipmann, Proc. Natl. Acad. Sci. USA 81 (1984) 3695–3698.
[25] U.K. Laemmli, Nature 227 (1970) 680–685.
[26] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.