WWOX Oxidoreductase – Substrate and Enzymatic Characterization

Anna Sałuda-Gorgul[a,§], Karolina Seta[b,§], Magdalena Nowakowska[b,§], and Andrzej K. Bednarek[b,*]

[a] Medical University of Lodz, Department of Analytical Chemistry, Muszynskiego 1, 90-151 Lodz, Poland
[b] Medical University of Lodz, Department of Molecular Cancerogenesis, Zeligowskiego 7/9, 90-752 Lodz, Poland. Fax: (+48) 42 768 24 65. E-mail: andrzej.bednarek@umed.lodz.pl

* Author for correspondence and reprint requests

Z. Naturforsch. **66 c**, 73–82 (2011); received May 12/September 14, 2010

WWOX is a tumour suppressor gene that spans the common fragile site FRA16D. Analysis of the *WWOX* expression pattern in normal human tissues showed the highest expression in testis, prostate, and ovary. Its altered expression has been demonstrated in different tissues and tumour types. The *WWOX* gene encodes a 414-amino acids protein, which is the first discovered protein with a short-chain dehydrogenase/reductase (SDR) central domain and two WW domains at the NH_2 terminus. Due to its potential role in sex-steroid metabolism, using two bacterial expression systems, we have cloned WWOX fusion proteins showing oxidoreductase activity in a crude extract, defined a course of enzymatic reactions for selected steroid substrates, and determined related K_m values. Our results show that the SDR domain of the WWOX protein has dehydrogenase activity and is reactive both in the presence of NAD^+ and $NADP^+$ for all examined steroid substrates. On the other hand, with the same substrates and reduced cofactors (NADH and NADPH) reduction activity was not observed.

Key words: WWOX, Oxidoreductase, Steroid Metabolism

Introduction

WWOX is a newly identified tumour suppressor gene located on a long arm of chromosome 16 (16q23.3-24.1) (Bednarek *et al.*, 2000). This genomic area is recognized as the common fragile site FRA16D, which is the second most affected fragile site in cancer. Both genomic alterations within the *WWOX* gene (Paige *et al.*, 2000, 2001) and its altered expression are frequently observed in different tissues and cancer types (Aqeilan *et al.*, 2004a; Guler *et al.*, 2004; Kuroki *et al.*, 2002, 2004; Nunez *et al.*, 2005a; Pluciennik *et al.*, 2006). Interestingly, it was shown that ectopically elevated *WWOX* expression in cancer cell lines results in tumour growth suppression and apoptosis (Bednarek *et al.*, 2001; Iliopoulos *et al.*, 2007; Kuroki *et al.*, 2004). A recent study has shown a possible regulation of *WWOX* expression by promoter methylation or histone deacetylation (Iliopoulos *et al.,* 2005, 2007; Ishii *et al.*, 2003). However, CpG island methylation of the *WWOX* gene did not display remarkable in prostate carcinomas and benign prostate hyperplasias (Bastian *et al.*, 2007). On the other hand, methylation analysis in pancreatic carcinogenesis demonstrated an increase in the expression of the *WWOX* gene after treatment with the methylation inhibitor 5-aza-2'-deoxycytidine (Kuroki *et al.*, 2004, 2006).

Analysis of the *WWOX* expression pattern in normal human tissues showed the highest expression in testis, prostate, and ovary, significantly lower expression in other tissues (spleen, thymus, small intestine or colon), and very low expression in bulk breast tissues (Bednarek *et al.*, 2000).

The *WWOX* gene encodes a 414-amino acids protein (46 kDa) which is the first discovered protein with a short-chain dehydrogenase/reductase (SDR) central domain and two WW domains at the NH_2 terminus (Bednarek *et al.*, 2000), and at the same time WWOX protein localization within the Golgi system requires an intact SDR domain (Bednarek *et al.*, 2001).

WW domains are involved in protein-protein interactions, and up to now several proteins were identified as candidates of WWOX partners. The WWOX protein interacts with p73 and this may result in redistribution of nuclear p73 to the cytoplasm, suppressing its transcriptional activity (Aqeilan *et al.*, 2004b). Moreover, the WWOX

§ These authors contributed equally to this work.

0939–5075/2011/0100–0073 $ 06.00

protein interacts with the Ap2-γ transcription factor involved in cell proliferation and suppresses its transactivating ability (Aqeilan *et al*., 2005). WWOX associates also with the full-length ErbB-4/tyrosine kinase receptor via its first domain, thus competes with the YAP protein, affecting its transcriptional activity (Aqeilan *et al.*, 2005).

Additionally, WWOX via its first WW domain specifically associates with the proline-rich motif of c-Jun proto-oncogene. Phosphorylation of c-Jun caused by Mekk1 (mitogen-activated protein kinase 1) enhances the interaction of c-Jun with WWOX. It was also found that expression of WWOX attenuates the ability of Mekk1 to increase the activity of c-Jun-driven AP-1 (activating protein 1).

Furthermore, the WWOX protein activity is regulated by Ack1 (Cdc42-intracellular associated tyrosine kinase), which phosphorylates WWOX at two different sites (Tyr^{33} and Tyr^{287}) resulting in two radically different outcomes (Mahajan *et al*., 2005).

Nunez *et al*. (2005a) observed a strong correlation between ER and PR with WWOX expression in breast cancer, but only the PR receptor status was associated with WWOX expression in ovarian carcinomas (Nunez *et al*., 2005b). These correlations were also studied by Pluciennik *et al*. (2006) in breast cancer. Such findings suggest that *WWOX* may be involved in steroid signalling/metabolic pathways through its SDR domain. The most conserved features of SDR proteins are two domains constituting the cofactor (GXXXGXG) and substrate (YXXXK) binding sites (Jornvall *et al*., 1995; Kallberg *et al*., 2002a). WWOX amino acid sequence analysis identified both the coenzyme [NAD(H) or NADP(H)] binding site GANSGIG at position 131–137 and the potential substrate binding site YNRSK at position 293–297 of the protein. Additionally, WWOX has a serine residue 12 amino acids upstream of the YNRSK substrate binding motif. This serine is at nearly identical location to that observed in steroid dehydrogenases (usually position – 13 from Tyr) (Bednarek *et al*., 2000; Duax and Ghosh, 1997). Moreover, based on clustering analysis of amino acid sequences of different SDR proteins, WWOX was recently classified in the same cluster as 17β-hydroxysteroid dehydrogenase 3 (Kallberg *et al*., 2002b; Marijanovic *et al*., 2003). Also Chang *et al*. (2005) showed that WWOX could be activated in cell culture conditions by 17β-estradiol treatment in lung epithelial cells, COS7 fibroblasts, and DU145 prostate cell line, but not in MCF7 breast cancer cell line. A strong association of *WWOX* expression with ER status reinforces the suggested role of this protein as an enzyme involved in sex-steroid metabolism (Nunez *et al*., 2005b). More recently, Aqeilan *et al*. (2009) confirmed on KO mice the role of WWOX in steroidogenesis and proper gonadal function. Its direct involvement in the steroidogenesis pathway was indicated by failure of Leidig cell formation, undectable levels of serum testosterone, reduced theca cells proliferation, and smaller follicles in ovary of KO mice, in which WWOX was absent or reduced. Moreover, they identified 15 genes involved in steroidogenesis which had altered expression levels in the absence of WWOX. They also showed that the first WW domain of this protein is essential for proper steroid-related gene expression and indicated the importance of this domain in the association process with regulating steroid enzymes transcription factors. These findings suggest that the WWOX protein participates in sex-steroid metabolism (Aqeilan *et al*., 2009).

In our study, using two bacterial expression systems, we have cloned WWOX fusion proteins showing oxidoreductase activity in a crude extract, defined a course of enzymatic reactions for selected steroid substrates, and determined related K_m values.

Experimental

Materials

WWOX cDNA in pcDNA 3.1.A vector came from A. K. Bednarek's collection; pET NusA Fusion System 44 was obtained from Novagen Inc. (Madison, WI, USA); TALON metal affinity resins (with Co^{2+}) were received from CLONTECH (Palo Alto, CA, USA); pGEX 2TK vector, a glutathione-S-transferase (GST) fusion protein expression vector, and BULK GST Purification Module were collected from Amersham Biosciences (Wien, Austria). CelLytic™ B II reagent, NAD^+, $NADP^+$, β-NADH, β-NADPH, 17β-estradiol (17β-E), estrone (E), 5α-androstane-3,17-dione (5α-A), 4-androstene-3,17-dione (4-A), progesterone (P), 5α-dihydroprogesterone-*allo* (5α-DHP-*allo*) were purchased from Sigma (St. Louis, MO, USA); testosterone (T) was bought from Fluka (Buchs, Switzerland); enzymes: BamHI, EcoRI, MssI,

SmaI, CIAP (calf intestine alkaline phosphatase), T4 DNA ligase were delivered from Fermentas UAB (Vilnius, Lithuania); Tween® 20, Nonident P-40 were taken from Calbiochem (Darmstadt, Germany); other reagents and organic solvents were obtained from Sigma and AppliChem GmbH (Darmstadt, Germany) or were of the highest quality commercially available.

Expression and soluble fraction with WWOX fusion protein

The *WWOX* cDNA fragment restricted by BamHI and EcoRI enzymes was subcloned from pcDNA 3.1.A into the pET 44a(+) expression vector. Constructs with WWOX-pET vector were subsequently introduced into *E. coli* TOP-10 and *E. coli* Origami B (DE3) pLysS by chemical transformation. For analysis of the WWOX protein, Origami B (DE3) pLysS cells, containing WWOX-pET or pET 44a(+) vector, were grown in 2 x YT medium at 37 °C with 50 μg/ml ampicillin. After the absorbance at 595 nm had reached 0.4–0.5, the cell cultures were chilled at 4 °C for 30 min. In order to induce protein expression isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM, ethanol to the final strength of about 0.2%, and cells were incubated overnight at 16–18 °C.

The cultures were centrifuged and resulting pellets washed with PBS buffer. Pellets then were lysed in mild conditions using CelLytic B II at room temperature. Subsequently, lysates were treated with lysozyme (final concentration of 120 μg/ml) for 5 min at 23 °C. Then RN-ase/DN-ase I mixture (8 μg/ml) and sarkosyl (0.003%) were added (incubation for 20 min at 23 °C). Finally, an equal volume of stabilizing buffer [40 mM Tris-HCl, pH 8.0, 0.6 M NaCl, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol, 0.4% Tween 20, and 0.4% Nonident P-40] was supplemented (incubation) for 30 min at 23 °C. Supernatants, containing a soluble fraction of NUS protein or NUS-WWOX fusion protein, were collected for analysis (Fig. 1A). Protein concentrations were measured according to Bradford (1976), using bovine serum albumin as a standard.

Fusion proteins purification was performed on glutathione-sepharose (Bulk GST Purification Module) and TALON metal affinity resin (with Co^{2+}) for GST-WWOX and NUS-WWOX fusion proteins, respectively (Figs. 1A, B). As the oxidoreductase activity was lost during purification, we have used a soluble fraction of a bacterial crude extract for enzyme kinetics analysis.

Initially, dehydrogenase activity was investigated using a common dehydrogenase substrate – *S*-indan-1-ol and NAD^+. Fig. 2 shows differences between oxidoreductase activity of NUS-WWOX and NUS (Fig. 2A), and GST-WWOX and GST proteins (Fig. 2B).

Enzyme assays

The dehydrogenase and reductase activities of a soluble fraction of the whole protein extract containing NUS protein or NUS-WWOX fusion protein were analysed using the following approach. All reactions were monitored at 340 nm at 25 °C to follow changes in absorbance of the nicotinamide nucleotide cofactor (ε = 6270 M^{-1} cm^{-1}) in time. The absorbance was measured in 5-min intervals, during a 30-min incubation with or without addition of $NAD(P)^+$ or NAD(P)H. The standard reaction mixture for both dehydrogenase and reductase activity consisted of 100 mM

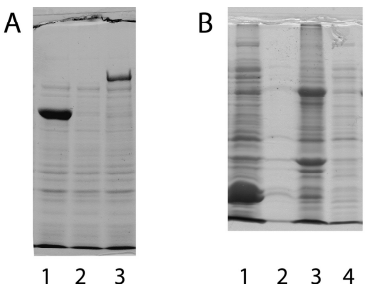


Fig. 1. Expression of the *WWOX* gene, as NUS-WWOX and GST-WWOX fusions, in *E. coli* cells. Electrophoretic separation was by SDS-PAGE, proteins were stained with Coomassie Brilliant Blue R-250. (A) 8% SDS-PAGE analysis of the induction of NUS-WWOX fusion protein. Lane 1, "NUS protein" soluble fraction of bacterial cell lysate with induction by 0.2 mM IPTG; lane 2, "NUS-WWOX fusion protein" soluble fraction of bacterial cell lysate without IPTG induction; lane 3, "NUS-WWOX fusion protein" soluble fraction of bacterial cell lysate with induction by 0.2 mM IPTG. (B) 12% SDS-PAGE analysis of the induction of GST-WWOX fusion protein. Lane 1, "GST protein" soluble fraction of bacterial cell lysate with induction by 0.7 mM IPTG; lane 2, "GST protein" soluble fraction of bacterial cell lysate without IPTG induction; lane 3, "GST-WWOX fusion protein" soluble fraction of bacterial cell lysate with induction by 0.7 mM IPTG; lane 4, "GST-WWOX fusion protein" soluble fraction of bacterial cell lysate without IPTG induction.

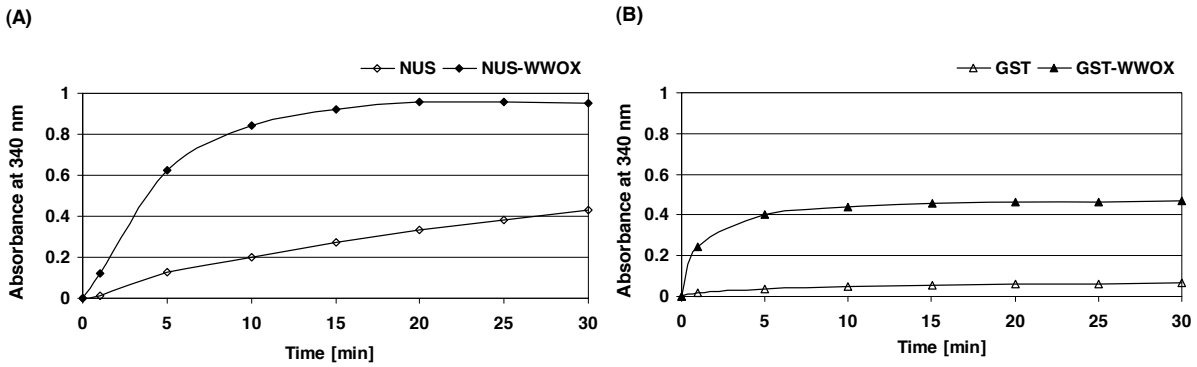


Fig. 2. Oxidoreductase activity in the soluble fractions of the crude protein extracts with WWOX fusion protein. Each assay was performed in relation to activity in the corresponding soluble fraction of the crude protein extract without WWOX. (A) Activities of two protein soluble fractions: "NUS-WWOX fusion protein" and "NUS protein" (Fig. 1A, lane 3 and lane 1, respectively). (B) Activities of "GST-WWOX fusion protein" and "GST protein", both soluble fractions (Fig. 1B, lane 3 and lane 1, respectively). The curves shown are from one of two experiments, each done with a separate isolation of the soluble fractions.

potassium phosphate buffer (pH 7.4), 0.75 μmol $NAD^+/NADP^+$ or 0.15 μmol NADH/NADPH, 0.24 μmol of each substrate (5α-androstane-3,17-dione, 4-androstene-3,17-dione, 17β-estradiol, estrone, 5α-dihydroprogesterone-*allo*, progesterone, testosterone), and 400 μg of the whole protein extract in a total volume of 1 ml. The reaction was initiated by addition of the whole protein fraction and corrected for initial changes.

The apparent K_m value for the steroid substrates was determined by the steady-state kinetics method, and Lineweaver-Burk analysis was applied in order to find K_m and V_{max} values for the WWOX dehydrogenase activity. Concentrations of the substrates were changed in 0.025-μmol/ml or 0.05-μmol/ml intervals (Table I). The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.4), 0.75 μmol NAD^+ or $NADP^+$, varying concentrations of the steroids, and 400 μg of the whole protein extract with NUS protein or NUS-WWOX fusion protein. After incubation at 25 °C steroid dehydrogenase assays were quenched with a 0.5-ml mixture of methanol/chloroform (1:1, v/v), vortexed, and the water fraction was separated by centrifugation. The dehydrogenase activity of the recombinant WWOX protein was calculated as the difference in absorbances at 340 nm of the NUS-WWOX fusion cell extract and plain NUS cell extract. Unless otherwise noted, the kinetics values were the means of at least two or three demonstrations.

Statistical analysis

Statistical analysis of the dehydrogenase activity levels of WWOX fusion protein in the crude bacterial protein extracts was performed using Mann-Whitney test. We compared K_m values for recombinant WWOX fusion protein (the difference between NUS-WWOX and NUS) with NUS

Table I. Concentrations of the steroid substrates for the enzymatic reactions with NAD^+ or $NADP^+$ as cofactors.

Substrate	Substrate concentration [μmol/ml]	
	Reaction with NAD^+	Reaction with $NADP^+$
5α-Androstane-3,17-dione (5α-A)	0.075 – 0.3	0.0375 – 0.225
4-Androstene-3,17-dione (4-A)	0.15 – 0.3	0.05 – 0.2
5α-Dihydroprogesterone-*allo* (5α-DHP-*allo*)	0.05 – 0.15	0.05 – 0.2
Progesterone (P)	0.05 – 0.15	0.05 – 0.2
17β-Estradiol (17β-E)	0.05 – 0.25	0.05 – 0.3
Estrone (E)	0.05 – 0.175	0.05 – 0.2
Testosterone (T)	0.075 – 0.225	0.075 – 0.225

cell extract K_m values and assumed the difference as significant when the confidence level was more than 95% ($p < 0.05$).

Results

NUS-WWOX and GST-WWOX fusion proteins extraction

Various variants of the purification process of NUS-WWOX fusion protein were investigated. Because the oxidoreductase activity was lost in any attempt of purification to near-homogeneity of WWOX fusion protein, we decided to use the soluble fraction of bacterial crude extract for our enzymatic studies.

In the second bacterial gene expression system we obtained a soluble fraction of the whole protein extract with GST-WWOX fusion protein, in which dehydrogenase activity was observed as well. The amount of the soluble GST-WWOX fusion protein was greater in the crude protein extract than in the preparations with NUS-WWOX, but its stability and the enzymatic activity varied over time. The same problem – loss of dehydrogenase activity – was observed during attempts of purification to homogeneity of the GST-WWOX fusion protein from bacterial proteins (Fig. 1).

Oxidoreductase activity of the WWOX protein

This work proves the oxidoreductase activity of the SDR domain, present in the NUS-WWOX fusion protein. Preliminary analysis of the redox reactions for various concentrations of steroid substrates, cofactors, and NUS-WWOX fusion protein in the crude protein extracts enabled us to determine conditions which were used for reactions in the presence of the steroids 5α-dihydroprogesterone-*allo* (5α-DHP-*allo*), progesterone (P), 5α-androstane-3,17-dione (5α-A), 4-androstene-3,17-dione (4-A), 17β-estradiol (17β-E), estrone (E), testosterone (T).

Because the enzymatic activity of NUS-WWOX fusion protein was studied in a crude extract of bacterial proteins, it was necessary to determine simultaneously the activities of crude protein extracts with NUS protein alone, in the presence of the same steroid substrates and cofactors. Endogenous oxidoreductase activities of whole protein extracts with NUS-WWOX fusion protein or only NUS protein without the steroid substrates for each cofactor were treated as a blank for each corresponding test.

Our results showed that the SDR domain of the WWOX protein has dehydrogenase activity and is reactive both in the presence of NAD^+ and $NADP^+$ for all examined steroid substrates. The reduction activities with the same seven steroid substrates and reduced cofactors (NADH or NADPH) were similar for both protein extract with NUS protein or NUS-WWOX fusion protein (results not shown), which indicates that the NUS-WWOX fusion protein has no reduction activity.

Dehydrogenase activity of SDR domain in the WWOX protein

A course of each enzymatic reaction was observed over the same period of time – 30 min for all steroids (5α-DHP-*allo*, P, 5α-A, 4-A, 17β-E, E, T) and NAD^+ or $NADP^+$ as a cofactor. For reactions with NAD^+ the amount of steroid hormone at 0.24 μmol/ml was sufficient or even excessive but the saturation of enzymatic proteins with the substrate occurred at different times. The oxidoreductase activity decreased dramatically when the specific steroid substrate was not added to the reaction mixture (Fig. 3).

For protein fractions with NUS-WWOX fusion protein, which had dehydrogenase activity, hyperbolic curves were obtained, suggesting that there is only one site for the steroid-to-substrate bond.

The enzymatic activity of the host bacterial protein extracts varied among substrates. The activity level was relatively low with 5α-DHP-*allo*, E, and T (3- to 6-fold lower compared to NUS-WWOX extracts), and for the remaining studied steroid substrates (P, 17β-E, 5α-A, 4-A) the oxidation activity was at the level corresponding to the blank.

For $NADP^+$ used as cofactor, reaction kinetics was similar to NAD^+ (Fig. 3B).

Our results obtained for the dehydrogenase activity of the WWOX fusion protein indicate that the WWOX protein was active with all seven steroids, and velocities of enzymatic reactions were higher for NAD^+ than $NADP^+$ as cofactor.

Subsequently K_m values for the dehydrogenase activity of the WWOX protein (as NUS-WWOX fusion protein) with the examined steroid substrates and cofactors were determined (Table II). For the given substrate, the enzymatic activity of the WWOX protein was calculated as the difference between activity of the crude protein extract with NUS-WWOX and the activity of the crude

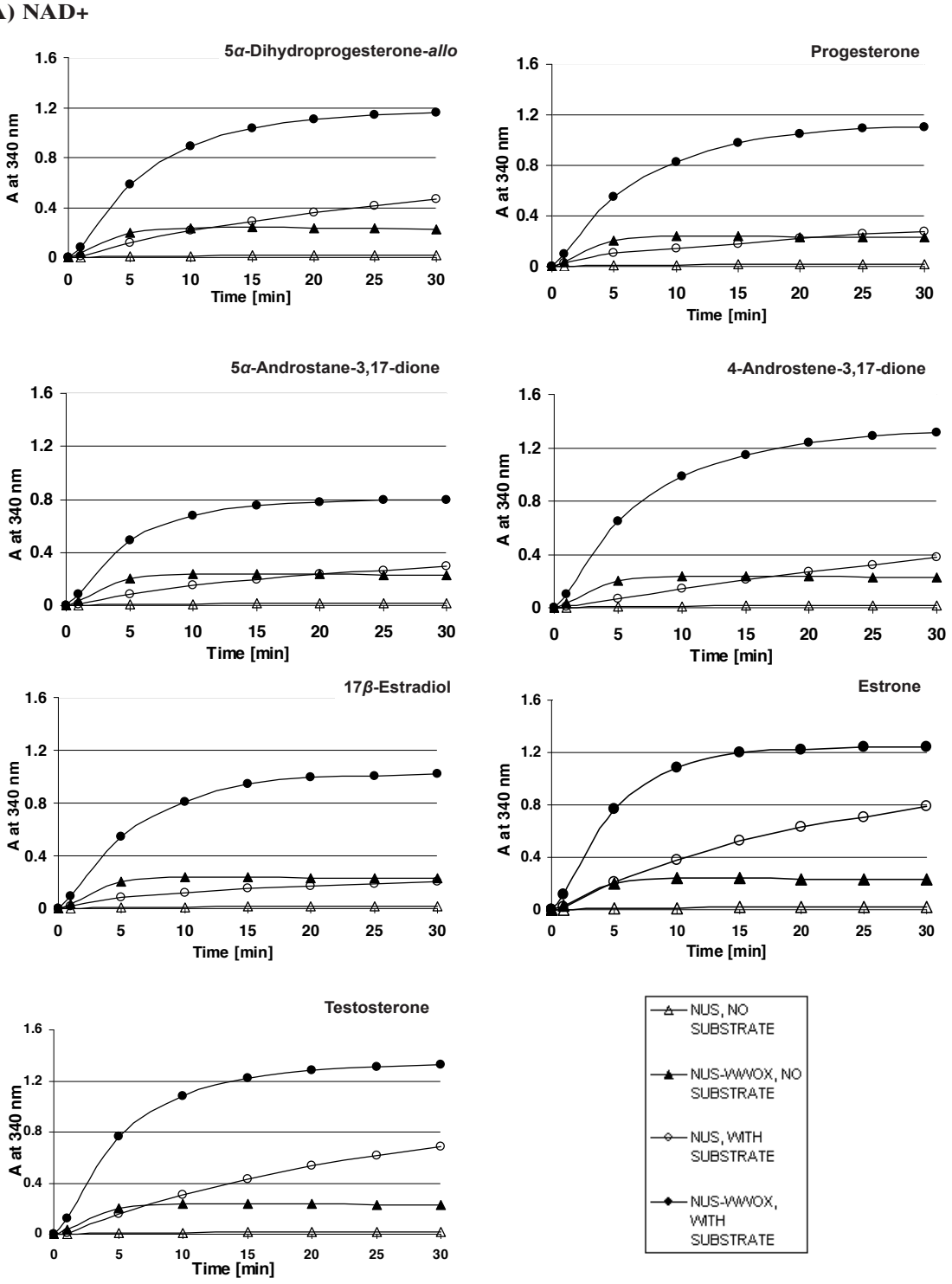
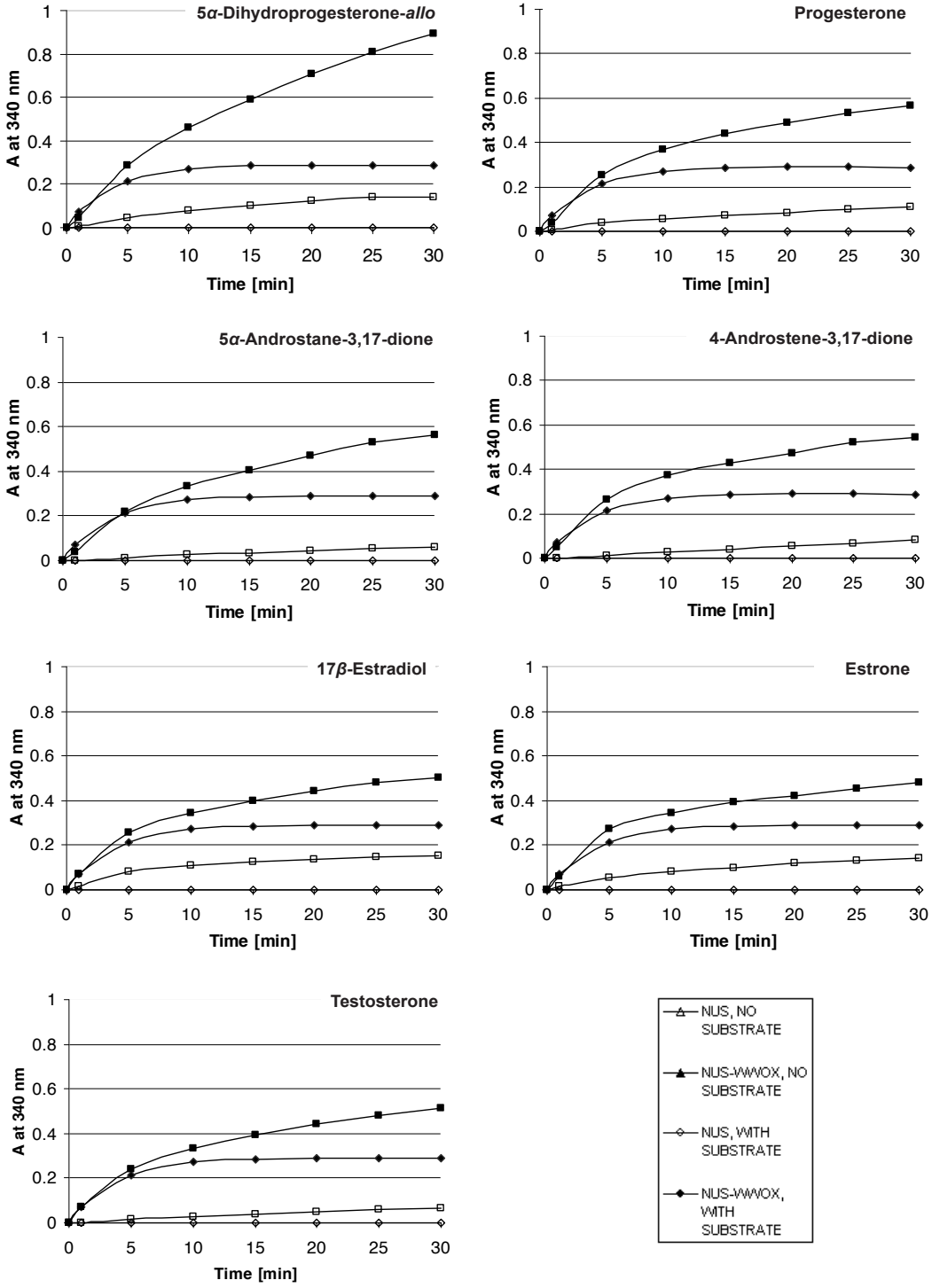


Fig. 3. Dehydrogenase activity of NUS-WWOX fusion protein with steroid substrates and (A) NAD^+ or (B) $NADP^+$ as cofactor. The absorbance of each assay was measured for four samples. Two of them were containing NUS-

(B) NADP+



WWOX fusion protein with or without steroid hormone, and the other two were consisting of NUS protein in the presence or absence of substrate.

Table II. Comparison of the K_m values of the NUS-WWOX fusion protein for chosen substrates.

Substrate	NAD^+			$NADP^+$		
	K_m [$\cdot 10^{-5}$ M]	C.I. for 95%	$p < 0.05$	K_m [$\cdot 10^{-5}$ M]	C.I. for 95%	$p < 0.05$
5α-Androstane-3,17-dione (5α-A)	5.864	4.42 – 8.72	0.0011	2.620	2.36 – 2.94	0.0000
4-Androstene-3,17-dione (4-A)	3.632	2.79 – 5.20	0.0006	3.703	2.81 – 5.43	0.0003
17β-Estradiol (17β-E)	3.123	2.62 – 3.87	0.0002	3.359	1.96 – 13.79	0.0091
Estrone (E)	1.523	1.11 – 2.42	0.0009	1.998	1.47 – 3.13	0.0007
5α-Dihydroprogesterone-*allo* (5α-DHP-allo)	3.985	2.80 – 6.93	0.0016	4.702	3.24 – 8.56	0.0017
Progesterone (P)	4.219	2.82 – 8.34	0.0023	3.021	2.48 – 3.92	0.0001
Testosterone (T)	4.161	3.13 – 6.19	0.0010	14.551	8.40 – 54.47	0.0025

C.I., confidence interval; p, significance level of Mann-Whitney test; K_m values are the difference between NUS-WWOX and NUS.

protein extract with NUS protein alone. This difference was obtained for the same conditions and concentrations of the reaction mixture. During spectrophotometric analysis the measure of this activity was the amount of NAD(P)H cofactor formed during the reaction. The lowest K_m values for the WWOX protein were obtained for estrone as steroid substrate ($1.523 \cdot 10^{-5}$ M with NAD^+ and $1.998 \cdot 10^{-5}$ M with $NADP^+$), whereas the highest were obtained for 5α-androstane-3,17-dione ($5.864 \cdot 10^{-5}$ M) and testosterone ($14.551 \cdot 10^{-5}$ M) with NAD^+ and $NADP^+$, respectively.

Discussion

WWOX is the first discovered protein which contains a SDR domain and two WW protein-protein interaction domains (Bednarek *et al.*, 2000). In the presented work the enzymatically active WWOX protein was obtained as a fusion protein in two bacterial expression systems. However, it appeared impossible to purify the fusion protein without loss of activity. This suggests that the WWOX protein with dehydrogenase activity is *in vivo* present in a cell only in liaison with other cell proteins which have to be substituted even in bacterial expression systems. In our study the WWOX protein retained its activity in the soluble fraction of a bacterial crude extract which may imply that WWOX partners are present amongst bacterial proteins.

In this experiment we proved that the SDR domain of the WWOX protein has dehydrogenase activity, and WWOX is reactive in the presence of all seven examined steroid substrates (5α-DHP-*allo*, P, 5α-A, 4-A, 17β-E, E, T) and NAD^+ and $NADP^+$ as cofactors. However, the activity of all substrates was higher with NAD^+.

On the other hand, with the same substrates and reduced cofactors (NADH and NADPH) reduction activity was not observed.

The highest expression of *WWOX* in hormone-dependent tissues, such as testis, prostate, and ovary, suggests its involvement in the steroid hormone metabolism. Indeed, this study confirms that the examined steroids may be the substrates for the SDR domain of the WWOX protein. Also the lowest K_m values of the WWOX protein obtained for estrone as steroid substrate suggests that the WWOX protein participates in the estrone metabolism *in vivo*. Moreover, the SDR domain in the WWOX protein has a specific amino acids

sequence (Bednarek *et al*., 2000) that was classified in the same cluster as 17β-hydroxysteroid dehydrogenase 3 (Kallberg *et al*., 2002b; Marijanovic *et al*., 2003). Our results are consistent with those from Chang *et al*. (2005) who showed that WWOX could be activated in cell culture conditions by 17β-estradiol treatment in lung epithelial cells and DU145 prostate cell line. Furthermore, is has been shown that the WWOX protein is necessary for proper gonadal development and function, and its absence alters the expression level of 15 steroidogenesis- related genes (Aqeilan *et al*., 2009). A correlation of ER and/or PR levels with *WWOX* expression was observed in breast and ovary cancers (Chang *et al*., 2005; Nunez *et al*., 2005a, b; Pluciennik *et al*., 2006), and this also supports the suggested role of this protein as an enzyme of central role in sex-steroid synthesis and metabolism.

Like in tumour cells incorrect WWOX transcripts have been found (Bednarek *et al*., 2000; Driouch *et al*., 2002), which lacked a substrate-binding part of the SDR domain; it is very likely that oxidoreductase activity and its loss in WWOX protein is of great importance in cancerogenesis. Therefore, further investigations concerning WWOX and its enzymatic activity need to be conducted.

Aqeilan R. I., Kuroki T., Pekarsky Y., Albagha O., Trapasso F., Baffa R., Huebner K., Edmonds P., and Croce C.M. (2004a), Loss of WWOX expression in gastric carcinoma. Clin. Cancer Res. **10**, 3053–3058.

Aqeilan R. I., Pekarsky Y., Herrero J. J., Palamarchuk A., Letofsky J., Druck T., Trapasso F., Han S. Y., Melino G., Huebner K., and Croce C. M. (2004b), Functional association between Wwox tumor suppressor protein and p73, a p53 homolog. Proc. Natl. Acad. Sci. USA **101**, 4401–4406.

Aqeilan R. I., Donati V., Palamarchuk A., Trapasso F., Kaou M., Pekarsky Y., Sudol M., and Croce C. M. (2005), WW domain-containing proteins, WWOX and YAP, compete for interaction with ErbB-4 and modulate its transcriptional function. Cancer Res. **65**, 6764–6772.

Aqeilan R. I., Hagan J. P., de Bruin A., Rawahneh M., Salah Z., Gaudio E., Siddiqui H., Volinia S., Alder H., Lian J. B., Stein G. S., and Croce C. M. (2009), Targeted ablation of the WW domain-containing oxidoreductase tumor suppressor leads to impaired steroidogenesis. Endocrinology **150**, 1530–1535.

Bastian P. J., Ellinger J., Heukamp L. C., Kahl P., Muller S. C., and von Rucker A. (2007), Prognostic value of CpG island hypermethylation at PTGS2, RAR-beta, EDNRB, and other gene loci in patients undergoing radical prostatectomy. Eur. Urol. **51**, 665–674.

Bednarek A. K., Laflin K. J., Daniel R. L., Liao Q., Hawkins K. A., and Aldaz C. M. (2000), WWOX, a novel WW domain-containing protein mapping to human chromosome 16q23.3-24.1, a region frequently affected in breast cancer. Cancer Res. **60**, 2140–2145.

Bednarek A. K., Keck-Waggoner C. L., Daniel R. L., Laflin K. J., Bergsagel P. L., Kiguchi K., Brenner A. J., and Aldaz C. M. (2001), WWOX, the FRA16D gene, behaves as a suppressor of tumor growth. Cancer Res. **61**, 8068–8073.

Bradford M. M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254.

Chang N. S., Schultz L., Hsu L. J., Lewis J., Su M., and Sze C. I. (2005), 17β-Estradiol upregulates and activates WOX1/WWOXv1 and WOX2/WWOXv2 *in vitro*: potential role in cancerous progression of breast and prostate to a premetastatic state *in vivo*. Oncogene **24**, 714–723.

Driouch K., Prydz H., Monese R., Johansen H., Lidereau R., and Frengen E. (2002), Alternative transcripts of the candidate tumor suppressor gene, WWOX, are expressed at high levels in human breast tumors. Oncogene **21**, 1832–1840.

Duax W. L. and Ghosh D. (1997), Structure and function of steroid dehydrogenases involved in hypertension, fertility, and cancer. Steroids **62**, 95–100.

Guler G., Uner A., Guler N., Han S. Y., Iliopoulos D., Hauck W. W., McCue P., and Huebner K. (2004), The fragile genes FHIT and WWOX are inactivated coordinately in invasive breast carcinoma. Cancer **100**, 1605–1614.

Iliopoulos D., Guler G., Han S. Y., Johnston D., Druck T., McCorkell K. A., Palazzo J., McCue P. A., Baffa R., and Huebner K. (2005), Fragile genes as biomarkers: epigenetic control of WWOX and FHIT in lung, breast and bladder cancer. Oncogene **24**, 1625–1633.

Iliopoulos D., Fabbri M., Druck T., Qin H. R., Han S. Y., and Huebner K. (2007), Inhibition of breast cancer cell growth *in vitro* and *in vivo*: effect of restoration of Wwox expression. Clin. Cancer Res. **13**, 268–274.

Ishii H., Vecchione A., Furukawa Y., Sutheesophon K., Han S. Y., Druck T., Kuroki T., Trapasso F., Nishimura M., Saito Y., Ozawa K., Croce C. M., Huebner K., and Furukawa Y. (2003), Expression of FRA16D/WWOX and FRA3B/FHIT genes in hematopoietic malignancies. Mol. Cancer Res. **1**, 940–947.

Jornvall H., Persson B., Krook M., Atrian S., Gonzalez-Duarte R., Jeffery J., and Ghosh D. (1995), Short-chain dehydrogenases/reductases (SDR). Biochemistry **34**, 6003–6013.

Kallberg Y., Oppermann U., Jornvall H., and Persson B. (2002a), Short-chain dehydrogenases/reductases (SDRs). Eur. J. Biochem. **269**, 4409–4417.

Kallberg Y., Oppermann U., Jornvall H., and Persson B. (2002b), Short-chain dehydrogenase/reductase (SDR) relationships: a large family with eight clusters common to human, animal, and plant genomes. Protein Sci. **11**, 636–641.

Kuroki T., Trapasso F., Shiraishi T., Alder H., Mimori K., Mori M., and Croce C. M. (2002), Genetic alterations of the tumor suppressor gene WWOX in esophageal squamous cell carcinoma. Cancer Res. **62**, 2258–2260.

Kuroki T., Yendamuri S., Trapasso F., Matsuyama A., Aqeilan R. I., Alder H., Rattan S., Cesari R., Nolli M. L., Williams N. N., Mori M., Kanematsu T., and Croce C. M. (2004), The tumor suppressor gene WWOX at FRA16D is involved in pancreatic carcinogenesis. Clin. Cancer Res. **10**, 2459–2465.

Kuroki T., Tajima Y., Furui J., and Kanematsu T. (2006), Common fragile genes and digestive tract cancers. Surg. Today **36**, 1–5.

Mahajan N. P., Whang Y. E., Mohler J. L., and Earp H. S. (2005), Activated tyrosine kinase Ack1 promotes prostate tumorigenesis: role of Ack1 in polyubiquitination of tumor suppressor Wwox. Cancer Res. **65**, 10514–10523.

Marijanovic Z., Laubner D., Moller G., Gege C., Husen B., Adamski J., and Breitling R. (2003), Closing the gap: identification of human 3-ketosteroid reductase, the last unknown enzyme of mammalian cholesterol biosynthesis. Mol. Endocrinol. **17**, 1715–1725.

Nunez M. I., Ludes-Meyers J., Abba M. C., Kil H., Abbey N. W., Page R. E., Sahin A., Klein-Szanto A. J., and Aldaz C. M. (2005a), Frequent loss of WWOX expression in breast cancer: correlation with estrogen receptor status. Breast Cancer Res. Treat. **89**, 99–105.

Nunez M. I., Rosen D. G., Ludes-Meyers J. H., Abba M. C., Kil H., Page R., Klein-Szanto A. J., Godwin A. K., Liu J., Mills G. B., and Aldaz C. M. (2005b), WWOX protein expression varies among ovarian carcinoma histotypes and correlates with less favorable outcome. BMC Cancer **5**, 64.

Paige A. J., Taylor K. J., Stewart A., Sgouros J. G., Gabra H., Sellar G. C., Smyth J. F., Porteous D. J., and Watson J. E. (2000), A 700-kb physical map of a region of 16q23.2 homozygously deleted in multiple cancers and spanning the common fragile site FRA16D. Cancer Res. **60**, 1690–1697.

Paige A. J., Taylor K. J., Taylor C., Hillier S. G., Farrington S., Scott D., Porteous D. J., Smyth J. F., Gabra H., and Watson J. E. (2001), WWOX: a candidate tumor suppressor gene involved in multiple tumor types. Proc. Natl. Acad. Sci. USA **98**, 11417–11422.

Pluciennik E., Kusinska R., Potemski P., Kubiak R., Kordek R., and Bednarek A. K. (2006), WWOX – the FRA16D cancer gene: expression correlation with breast cancer progression and prognosis. Eur. J. Surg. Oncol. **32**, 153–157.