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Investigation of a potential role for aldose reductase AlrA in tetrahydropteridine synthesis in *Dictyostelium discoideum* Ax2

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Abstract: *Dictyostelium discoideum* Ax2 is well-known for the synthesis of D-threo-tetrahydrobiopterin (DH4) with a smaller amount of L-erythro-tetrahydrobiopterin (BH4). DH4 synthesis from 6-pyruvoyltetrahydropterin (PPH4) is catalyzed by aldose reductase (AR)-like protein and sepiapterin reductase (SR) via an intermediate 1′-oxo-2′-D-hydroxypropyl tetrahydropterin, which is non-enzymatically oxidized to D-sepiapterin in the absence of SR. However, L-sepiapterin was a dominant product in the reaction of a cellular extract of *spr*$^-$ disrupted in the SR gene. In order to investigate its potential role in tetrahydropteridine synthesis, the enzyme catalyzing L-sepiapterin synthesis from PPH4 was purified from *spr*$^-$. Via mass spectrometry, the protein was identified to be encoded by *alrA*. AlrA consists of 297 amino acid residues sharing a high sequence identity with human AR. However, in the co-incubation assay, DH4 synthesis was not detected and, furthermore, the recombinant AlrA was observed to suppress BH4 synthesis by SR, which was known to prefer 1′-oxo-2′-D-hydroxypropyl tetrahydropterin to PPH4. Although intracellular DH4 level in *alrA*$^-$ was decreased to 60% of the wild type, it is presumed to result from the antioxidant function of DH4. Therefore, despite the structural and catalytic identities with human AR, AlrA seems to be involved in neither BH4, nor DH4 synthesis under normal physiological conditions.

Keywords: aldose reductase; antioxidant; *Dictyostelium*; sepiapterin reductase; tetrahydrobiopterin.

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Introduction

L-erythro-tetrahydrobiopterin (BH4) is a multifunctional molecule playing the role of an antioxidant and a cofactor for aromatic amino acid hydroxylation, nitric oxide synthesis and alkylglycerol hydroxylation in mammals [1]. The de novo biosynthesis of BH4 in mammals starts from guanosine 5′-triphosphate (GTP) by sequential catalyses of GTP cyclohydrolase I (GTPCH; EC 3.5.4.16), 6-pyruvoyltetrahydropterin synthase (PTPS; EC 4.2.3.12), and sepiapterin reductase (SR; 1.1.1.153) [1]. GTPCH catalyzes the synthesis of dihydroneopterin triphosphate (H_2-NTP), which is transformed to 6-pyruvoyltetrahydropterin (PPH4) by PTPS. The diketo compound PPH4 converts to BH4 by SR via two consecutive reductions of the C1′ carbonyl group involving an isomerization reaction [2]. In addition to SR two human enzymes, aldose reductase (AR; EC 1.1.1.21) and AKR1C3 (EC 1.3.1.20) belonging to the aldo-keto reductase (AKR) superfamily are involved in the BH4 synthesis [3–5]. AR catalyzes C2′-specific reduction of PPH4 to 1′-oxo-2′-L-hydroxypropyl tetrahydropterin, which is further reduced to BH4 by SR. AKR1C3 catalyzes the C1′-specific reduction of PPH4 to 1′-hydroxy-2′-oxopropyl tetrahydropterin, which can be reduced further to BH4 by AR. Therefore, collaboration of both AKRs can generate BH4 from PPH4, constituting an alternative pathway of the BH4 synthesis in the absence of SR [5, 6].

A multicellular eukaryote *Dictyostelium discoideum* Ax2, a useful model organism for cellular and developmental research, is well known for its synthesis of two isomeric forms of tetrahydropteridines: BH4 and its D-threo isomer (tetrahydrodictyopterin, DH4) [7]. Both tetrahydropteridines are reported to have dual functions of an antioxidant and a cofactor for phenylalanine hydroxylase [8, 9], although it remains unclear whether their coexistence has any physiological significance [10]. *Dictyostelium* is also similar to mammals in the biosynthesis of BH4 and DH4 [11]. While BH4 can be generated from PPH4 by *Dictyostelium* SR (dicSR) alone [12], the DH4 synthesis needs a collaborative work of dicSR and another protein, which was named AR-like protein due to its catalytic similarity to AR [11]. As the AR-like protein was

presumed to convert PPH4 to 1′-oxo-2′-D-hydroxypropyl tetrahydropterin, which would be non-enzymatically oxidized to D-sepiapterin in the absence of dicSR, we naturally expected to find D-sepiapterin in the aerobic assay conditions. By contrast, however, we found in this study that L-sepiapterin is dominant over D-sepiapterin in the reaction mixture of the crude extract prepared from the knockout mutant of the SR gene. In order to study the functional significance of the L-sepiapterin production for tetrahydropteridine synthesis in *D. discoideum* Ax2, we purified the enzyme responsible for the activity and found that it is encoded by *alrA* gene in the Ax2 genome. We also investigated the catalytic properties of the protein through both *in vitro* and *in vivo* experiments and report here the results.

Materials and methods

Cell growth

The knockout mutant of the SR gene (*spr*$^-$) was prepared from the result reported elsewhere [11]. The *alrA* knockout mutant (*alrA*$^-$), which was created originally by Prof. Gomer's laboratory [13], was obtained from the Dictybase (http://dictybase.org/). The cells were grown vegetatively at 22°C in the HL5 medium (10 g glucose, 10 g protease peptone, 7.15 g yeast extract, Na_2HPO_4 0.35 g, KH_2PO_4 0.35 g, pH 6.5, per liter) with 100 μg/mL streptomycin sulfate and 100 U/mL benzylpenicillin potassium [14].

cDNA cloning and protein expression

AlrA cDNA was amplified from the total RNA extracted from the Ax2 vegetative cells by using the TRI reagent (Molecular Research Center. Inc). cDNA was prepared from 2 μg of total RNA by using QIAGEN OneStep RT-PCR Kit (QIAGEN). The full-length ORF sequence of AlrA was amplified by PCR from the cDNA using the primer pair 5′-gctagcatggaaccatcatttaaattatcatctg-3′ (AlrA-F), 5′-ggatccttaattgaaaagtggtacaccccag-3′ (AlrA-R). The amplified DNAs cloned into pGEM-T vector were digested with NheI/BamHI and subsequently cloned into the corresponding restriction sites of pET-28b. The PCR amplifications were performed with *Pfu* polymerase in 1× reaction buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM each of primer pairs, and templates, under the following conditions: 4 min at 95°C, followed by 30 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min, and a final polymerization at 72°C for 10 min. The plasmid was transformed into the BL21(DE3) competent cells. *Escherichia coli* BL21(DE3) transformant was induced with 0.1 mM isopropyl-b-D-thiogalactopyranoside and was cultured overnight at 30°C. The recombinant proteins containing N-terminal 6xHis-tag were purified by chromatography on Ni-nitrilotriacetic acid gel according to the manufacturer's (Qiagen) instructions. The proteins were eluted with 250 mM imidazole, dialyzed against a mixture of 20 mM Tris-SDS HCl (pH 7.5) and 30% (vol/vol) glycerol, and stored in aliquots at 70°C until further use. Purification of the proteins was confirmed by electrophoresis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel.

Protein purification and identification

In order to avoid interference by indigenous SR, *spr*$^-$ cells were used for isolation of the AlrA protein. Harvested cells (3 g wet weight) were suspended in 9 mL of homogenization buffer [20 mM Tris-HCl, pH 8.5, 1X protease inhibitor cocktail (Roche Life Sci.), and 5% glycerol] and sonicated with Vibra Cell (Sonics and Materials, USA). The homogenate was centrifuged for 20 min at 18,000×*g* to discard the precipitate and then filtered through 0.2 μm syringe filter. The supernatant was loaded on a column (1×6.7 cm) of Affi-gel blue preequilibrated with buffer A (20 mM Tris-HCl, pH 8.5, 5% glycerol) and eluted with the linear gradient of 0–500 mM NaCl in 24 mL of buffer A at the flow rate of 1 mL/min. The active fractions were combined and then concentrated by ultrafiltration through a Spin-XR UF 500 (Corning, UK). The enzyme solution was applied to a Superdex 75 10/300 GL (Amersham Biosciences, Sweden) preequilibrated with buffer A and chromatographed with the same buffer at the flow rate of 0.8 mL/min. The active fractions were applied to a Mono Q 5/50 GL (Amersham Biosciences, Sweden) and eluted with the linear gradient of 0–150 mM NaCl in 39 mL of buffer A at the flow rate of 1 mL/min. The active fractions were concentrated and applied to a Superdex 200 10/300 GL (Amersham Biosciences, Sweden) preequilibrated with buffer A and chromatographed with the same buffer at the flow rate of 0.5 mL/min. The active fractions were analyzed for purity by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue G-250. The protein was measured by the Bradford method, using bovine serum albumin as a standard.

For protein identification, the SDS-PAGE gel fragment of 35 kDa was subjected to Electrospray ionization tandem mass spectrometry (ESI-MS/MS), which was performed by eMass (http://www.emass.co.kr, Korea).

Enzyme activity assay

Unless described otherwise, all reactions were carried out in the reaction volume of 50 μL containing 50 mM Tris-HCl (pH 8.5), 0.2 mM NADPH, 1 mM dithiothreitol, 10 mM $MgCl_2$, aliquots of H_2-NTP, 50 ng of recombinant human PTPS, and other enzymes. The reaction mixture was incubated at 22°C for 1 h. For the determination of sepiapterin the reaction was stopped by incubation at 95°C for 5 min and centrifuged for the HPLC analysis. For the determination of biopterin and dictyopterin, the reaction was terminated by mixing with an equal volume of acidic iodine solution (2% KI and 1% I_2 in I N HCl) [15]. After 1 h in the dark, the centrifugal supernatant was mixed in a 10:1 volume ratio with 5% ascorbic acid and subjected to HPLC. H_2-NTP was prepared from GTP by incubation with the recombinant *Synechocystis* sp. PCC 6803 GTPCH [16]. The recombinant proteins of dicSR, human SR and human PTPS were prepared as described elsewhere [12, 17, 18].

For the identification of enantiomeric forms of sepiapterin, the heat-inactivated reaction mixture was incubated with 1 μg of dicSR and 0.2 mM NADPH at 37°C for 30 min to convert sepiapterin to dihydrobiopterin. After acidic iodine oxidation, the fully oxidized D-threo form (dictyopterin) and the L-erythro form (biopterin) were separated by HPLC.

HPLC was performed with a C18 column (Agilent HC-C18, 5 μm, 4.6×150 mm) and a fluorescence detector (Shimadzu model RF-10A$_{XL}$) at the constant flow rate of 1.2 mL/min. Sepiapterin was eluted isocratically with 10 mM potassium phosphate (pH 6.0) containing 8% methanol and monitored at 425 nm/530 nm (ex/em). Other pteridines were eluted isocratically with 10 mM potassium phosphate (pH 6.0) and monitored at 350 nm/450 nm (ex/em). Authentic pteridines were purchased from Dr. Schircks Laboratories (Jona, Switzerland).

The HPLC data were collected from three replicates and are expressed as the mean ± SD, while the chromatograms are representative ones. Statistical analysis was performed using a paired Student's t-test.

Results and discussion

The catalytic function of AR-like protein to convert PPH4 to 1′-oxo-2′-D-hydroxypropyl tetrahydropterin was examined using a crude extract of *spr*$^-$ cells. In the co-incubation assay containing both the crude extract and recombinant dicSR, the HPLC analysis of the reaction mixture showed dictyopterin as well as biopterin (Figure 1, upper chromatogram), indicating the presence of AR-like protein in the crude extract [11]. Therefore, without dicSR, the reaction mixture was expected to show D-sepiapterin, which was originated from 1′-oxo-2′-D-hydroxypropyl tetrahydropterin. In order to convert sepiapterin to non-enantiomeric biopterin isomers, the reaction mixture was incubated with dicSR and then analyzed for the isomers by HPLC (Figure 1, lower chromatogram). Contrary to our expectations, biopterin was dominant over dictyopterin, indicating the overwhelming presence of L-sepiapterin, rather than D-sepiapterin, in the original reaction mixture. As the enzyme activity responsible for the L-sepiapterin production was never expected in *Dictyostelium* and, furthermore, dictyopterin was barely detectable in the reaction mixture, we decided to identify the enzyme via protein purification.

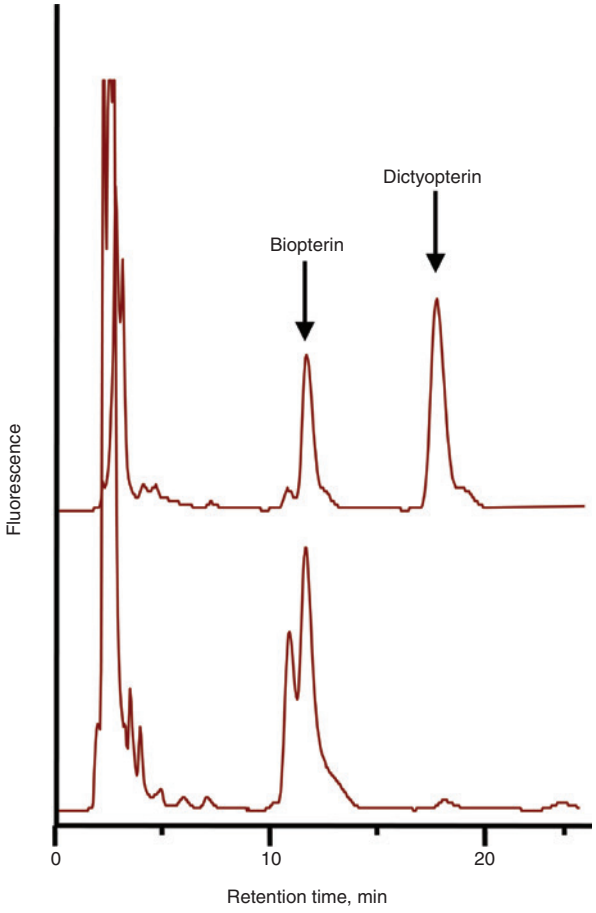

Figure 1: HPLC analysis of AR-like protein and AlrA activities in the crude extract of *spr*$^-$ cells.
Upper chromatogram: Co-incubation assay of the crude extract and dicSR. Lower chromatogram: Separate incubation assay of the crude extract and dicSR. dicSR was added after the crude extract reaction was terminated by heating. The reaction mixtures were analyzed after iodine oxidation. The detector sensitivity increased four-fold in the lower chromatogram in order to show the dictyopterin peak.

The enzyme catalyzing PPH4 to L-sepiapterin was purified from a crude extract of *spr*$^-$ cells using four successive chromatographic steps (Figure 2; Table 1 for a summary of the results). When the purified enzyme was subjected to SDS-PAGE, a major protein band corresponding to the molecular mass of ca. 35 kDa was observed (Figure 3A). In the final purification step on Superdex 200, the active fractions were eluted as a single peak at a volume corresponding to a molecular mass of ~75 kDa (Figure 2D), indicating that the native enzyme assembles as a dimer. The 35 kDa protein band in the SDS-PAGE gel was subjected to the ESI-MS/MS analysis to find several peptide fragments corresponding to those in the protein sequence encoded by *alrA*. The AlrA protein consists of 297 amino acid residues having a deduced molecular mass of 33,649 Da. AlrA also shared over 35% sequence identities with AR and AKR1C3 (Figure 3B), which are well known to catalyze reduction of PPH4 to 1′-oxo-2′-L-hydroxypropyl tetrahydropterin and 1′-hydroxy-2′-oxopropyl tetrahydropterin, respectively [6].

As a homolog of AR, it appeared to be clear that AlrA reduces PPH4 to 1′-oxo-2′-L-hydroxypropyl tetrahydropterin, which is eventually oxidized to L-sepiapterin under the aerobic assay conditions. In order to further examine the putative functional role of AlrA in the tetrahydropteridine synthesis, the His-tagged recombinant protein of AlrA prepared from *E. coli* was used for *in vitro* assay (Figure 4). The specific activity of the recombinant AlrA to produce L-sepiapterin from PPH4 was determined to

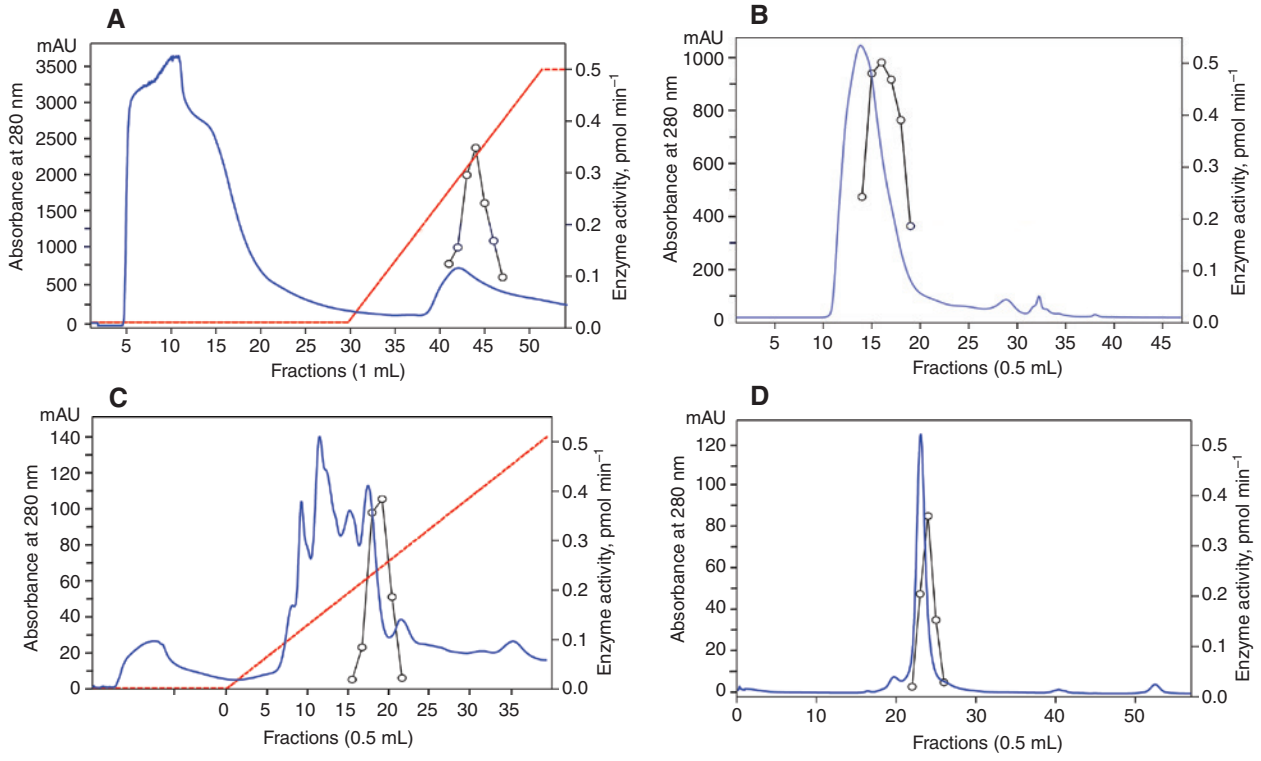

Figure 2: Purification of AlrA protein.
(A) Affi-gel blue chromatography (1×6.7 cm) with a linear gradient of 0–500 mM NaCl. (B) Superdex 75 10/300 GL chromatography. (C) Mono Q 5/50 GL chromatography with a linear gradient of 0–150 mM NaCl. (D) Superdex 200 10/300 GL chromatography. Solid blue line, protein; open circle dark line, enzyme activity; dotted red line, salt gradient.

Table 1: Summary of the purification of AlrA protein from *spr*⁻ cells.

Procedure	Total protein, mg	Total activity, pmol min⁻¹	Specific activity, pmol min⁻¹mg⁻¹	Yield, %	Purification fold
Crude extract	306.440	2283.2	7.5	100.0	1.0
Affi-gel blue	4.652	320.9	69.0	14.1	9.3
Superdex 75 10/300	2.378	363.4	152.8	0.2	20.5
MonoQ 5/50	0.112	123.4	1105.8	5.4	148.4
Superdex 200 10/300	0.032	59.9	1862.9	2.6	250.0

be 15.6±0.7 nmol/mg protein/min at 37°C, which is ca. 6 times lower than that of AR [5]. When AlrA was co-incubated with dicSR, DH4 synthesis was not detected. Furthermore, the dicSR activity to produce BH4 decreased remarkably, while the hSR activity did not decrease much (Figure 4A). The suppression effect of AlrA on dicSR activity was confirmed in the co-incubation assay repeated with varying amounts of AlrA (Figure 4B). It is presumed that AlrA is better than dicSR in catalyzing PPH4 and, furthermore, its enzymatic product 1′-oxo-2′-L-hydroxypropyl tetrahydropterin is poorly catalyzed by dicSR. In support of this assumption, it was suggested previously that dicSR prefers 1′-oxo-2′-D-hydroxypropyl tetrahydropterin over PPH4 [17].

To find out if there is any functional association of AlrA with *in vivo* synthesis of tetrahydropteridines, pteridine levels were determined in *alrA*⁻ cells (Figure 5). Compared to wild type, biopterin level increased by ca. 39% in the mutant, probably due to a higher availability of PPH4 for dicSR in the absence of AlrA, as shown in the co-incubation assay (Figure 4). On the other hand, dictyopterin level decreased by ca. 40% in the mutant, raising the question whether AlrA is somehow involved in the DH4 synthesis *in vivo*. However, the partial impact of *alrA* knockout on the DH4 synthesis, as well as the *in vitro* assay results (Figure 4), may support another possibility. Specifically, AKRs are well known for their broad spectrum of substrate specificity. AlrA was also shown to catalyze reduction of

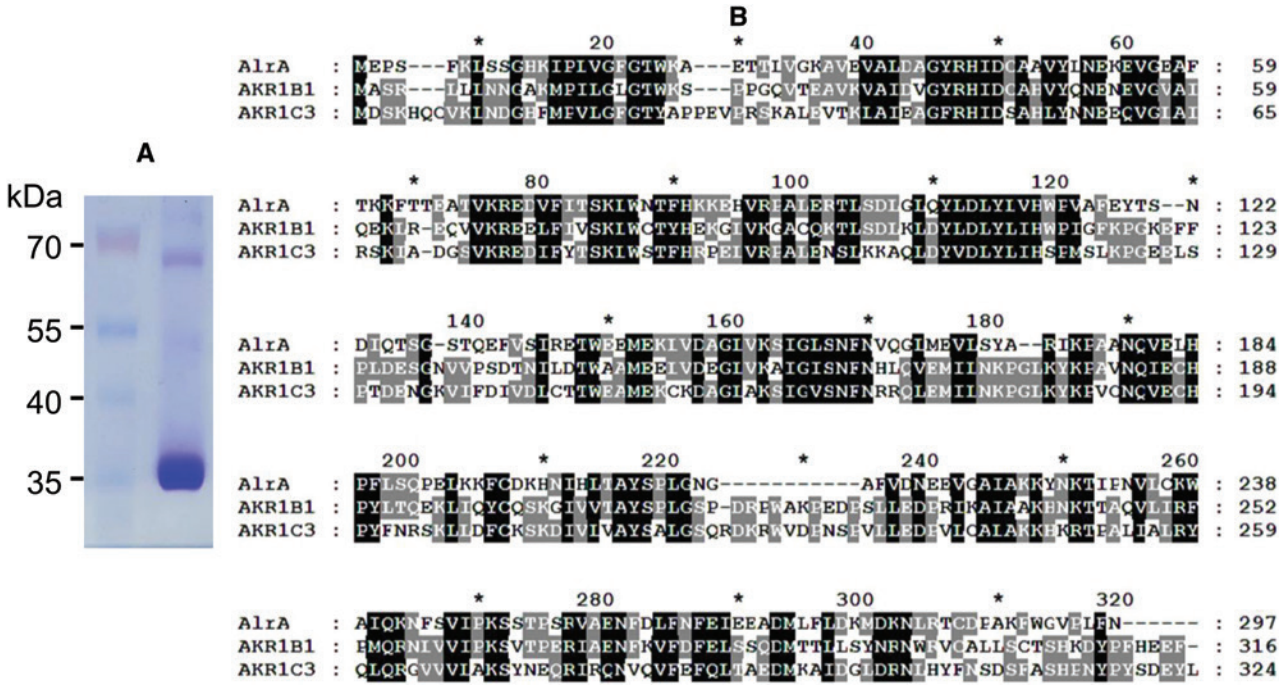

Figure 3: SDS-PAGE of the purified AlrA and its homology to human AKRs.
(A) The final purified fraction was analyzed by a SDS-PAGE of 10% polyacrylamide under reducing condition. The molecular masses of the marker proteins are shown on the left. The protein having an apparent molecular mass of 35 kDa was subjected to mass spectrometry.
(B) Multiple alignment of protein sequences. Conserved sequences are shaded at four levels using GeneDoc software.

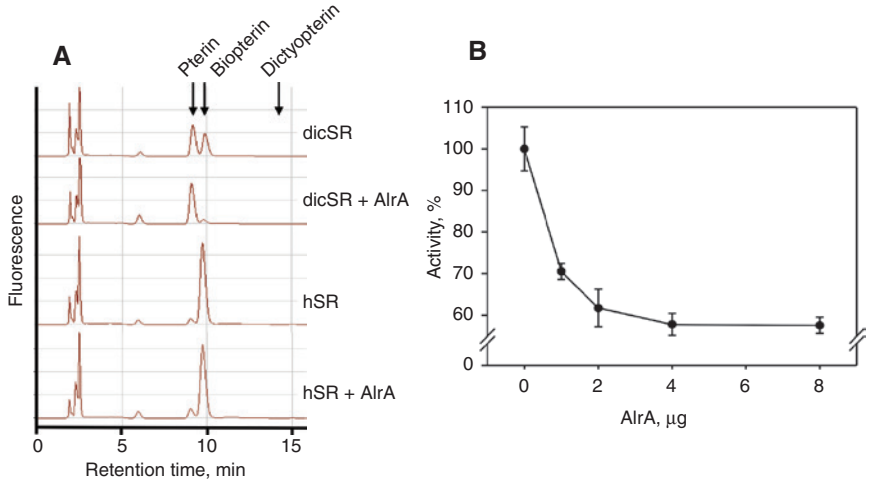

Figure 4: *In vitro* assay of the recombinant AlrA.
dicSR and hSR were incubated alone or co-incubated with AlrA. Equal amounts of proteins (2 µg each) were used for the assay. (A) The reaction mixtures analyzed by HPLC after acidic iodine oxidation. (B) BH4 production in the co-incubation assay where a fixed amount (2 µg) of dicSR or human SR was used.

methylglyoxal, a toxic endogenous α-ketoaldehyde, and knockdown of *alrA* stimulated accumulation of methylglyoxal [19]. An excess of methylglyoxal formation can increase reactive oxygen species production and cause oxidative stress [20]. Therefore, it may be highly plausible that oxidative stress in *alrA*⁻ cells might have contributed to the remarkable decrease in the amount of DH4, which is known to play a role of an antioxidant [8, 10].

In conclusion, this study is the first to show in *D. discoideum* Ax2 that there is a homolog of mammalian

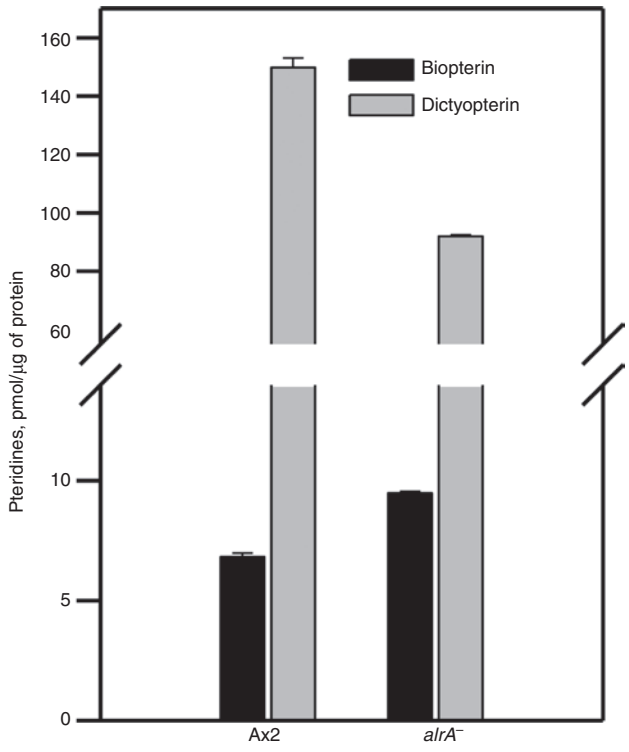

Figure 5: Comparative analysis of pteridine levels in wild type and *alrA*⁻ cells.
The cells grown in the HL5 medium were harvested and determined for total cellular pteridines as fully oxidized forms after acidic iodine oxidation. The two-tailed p values were <0.001.

AR, which catalyzes C2′-specific reduction of PPH4 to 1′-oxo-2′-L-hydroxypropyl tetrahydropterin. However, due to the peculiar substrate specificity of dicSR different from mammalian SRs, AlrA appears to be involved in neither BH4, nor DH4 synthesis under normal physiological conditions. Although there was a remarkable decrease in cellular DH4 level in *alrA*⁻, it is presumed to be associated with the antioxidant function of DH4. In addition, we also studied four homologs of AR (AlrB, AlrD, AlrE, and AlrF) in the Ax2 genome and found that, despite having an identical catalytic function to generate L-sepiapterin from PPH4 (unpublished experiments), they are less active than AlrA.

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