Homoserine kinase in *Chlamydomonas reinhardtii*: biochemical characterization and expression pattern

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Summary

Homoserine kinase (EC 2.7.1.39, HSK) produces O-phospho-L-homoserine, a branch point intermediate in the pathways for methionine (Met) and for threonine (Thr)/isoleucine (Ile) in photosynthetic eukaryotes. Although HSKs have been described in bacteria, archaea and higher plants, no HSK homolog has so far been characterized in green algae.

In the unicellular green alga *Chlamydomonas reinhardtii*, the HSK1 is encoded by a nuclear gene CrHSK1. From the analysis of the localizations demonstrated for homologous proteins in higher plants *Chlamydomonas* HSK is predicted as chloroplast protein. The N-terminal part of CrHSK1 until residue alanine 31 is predicted to encode a chloroplast transit peptide that is cleaved in the mature protein. The *C. reinhardtii* HSK1 (CrHSK1) was cloned and overexpressed with a C-terminal-fused His6-tag. Gel filtration showed the oligomeric structure of CrHSK1 to be a homodimer. CrHSK1 has an apparent Km for L-homoserine of 0.107 mM and for ATP of 0.2 mM and it required K⁺ and Mg²⁺ for activity. CrHSK1 appears to be insensitive to the substrate and end-products of the aspartate pathway it serves. In *C. reinhardtii*, CrHSK1 transcript levels are induced in response to peroxide treatment. These results suggest that CrHSK1 might be implicated in oxidative stress response. Characterization of HSK in the green alga provides a framework for a more complete understanding of the regulation of this highly conserved enzyme.

Key words: aspartate pathway, *Chlamydomonas reinhardtii*, gene expression, homoserine kinase, oxidative stress

Introduction

Like most free-living microorganisms, *Chlamydomonas reinhardtii* synthesizes the whole range of amino acids that are required to form proteins and most important metabolites (polyamines, nucleotides, glutathione etc.). *C. reinhardtii* cells carry out the bulk of their amino acid biosynthesis in the chloroplast. A series of amino acids are synthesized through the aspartate pathway. Similar to higher plants (Azevedo et al., 1997; Bryan, 1980; Jander and Joshi, 2009; 2010), the aspartate pathway in *C. reinhardtii* consists of three branches, one leading to lysine, another to threonine and...
isoleucine, and the third to methionine (Fig. 1). The branching point to lysine occurs at aspartate-4-semialdehyde (A4S) whereas at homoserine-4-phosphate (O-phospho-L-homoserine, OPH), the pathway branches to methionine and to threonine/isoleucine. It is notable that the pathway for biosynthesis of methionine from OPH in *C. reinhardtii* and in higher plants differs from that of bacteria (Vallon and Spalding, 2009). In prokaryotic microorganisms, the analogous branch-point occurs at homoserine, with OPH used specifically for threonine synthesis, and O-succinylhomoserine or O-acetylhomoserine used specifically for methionine synthesis (Greene, 1996; Patte, 1996).

O-phospho-L-homoserine synthesis is catalyzed by homoserine kinase (HSK; EC 2.7.1.39). It belongs to a large class of small metabolite kinases, the GHMP kinase superfamily (Zhou et al., 2000). The properties of HSK from prokaryotic microorganisms have been well documented (Miyajima and Shio, 1972; Thêze et al., 1974). Moreover, the three-dimensional structure of HSK from *Methanococcus jannaschii* has been described (Zhou et al., 2000). HSKs have also been characterized among the higher plant species (Aarenes, 1976; Azevedo et al., 1997), but evidences for their regulatory properties are variable. In contrast to studies showing that HSK from radish and pea are allosterically inhibited by threonine, isoleucine, valine and S-adenosyl-L-methionine (Baum et al., 1983; Thoen et al., 1978), the purified enzymes from wheat germ and *Arabidopsis* were found to be not influenced by these amino acids (Riesmeier et al., 1993; Lee and Leustek, 1999; Jander and Joshi, 2010). It was suggested that in monocotyledonous plants HSK is not a regulatory enzyme of the aspartic acid pathway (Riesmeier et al., 1993). Characterization of HSKs in green algae will provide a framework for a better understanding of the regulation of this key enzyme.

The unicellular green alga *Chlamydomonas* has long been one of the most successful eukaryotic phototrophic model microorganisms for genetic and biochemical studies of many cellular processes including nitrogen metabolism (Grossman and Takahashi 2001; Fernández and Galván, 2007; Ermilova et al., 2010). This has been possible because of the development of excellent biochemical, molecular, genetic and genomic tools. In addition, the *Chlamydomonas* Genome Sequence Project is promoting the use of this organism in functional and comparative genomic studies. This article provides the first characterization of a HSK in a representative photosynthetic eukaryotic microorganism. We examined the oligomeric structure, regulatory properties and expression patterns of HSK1 from *C. reinhardtii* (CrHSK1) as a model eukaryotic microorganism.

**Material and methods**

**Strains and growth conditions**

The strain cw15-325 (cw15 mt+arg7-8) was kindly provided by Dr. M. Schröda (University of Kaiserslautern, Germany). Cells were grown mixotrophically in Tris-acetate-phosphate (TAP) medium (Gorman and Levine, 1965) under continuous irradiation with white light (fluence rate of 45 µE m⁻² s⁻¹) at 22 °C. The TAP medium was supplemented with 50 mg/l of arginine. The number of cells was recorded microscopically with the use of a counting chamber.

**Cloning, expression and purification of HSK proteins**

For overexpression of His₅-tagged recombinant CrHSK1, an artificial gene with an optimized codon usage for *E. coli* expression was synthesized from Eurofins MWG Operon (Ebersberg, Germany). The DNA sequence was derived from the amino acid sequence of the mature, potentially chloroplast-localized CrHSK1 (starting with amino acid 31 (AGGKV) of the non-processed CrHSK1 sequence, accession number A81826). The synthetic gene in pEX-A (Eurofins MWG Operon, Ebersberg, Germany).
Germany) vector was digested with NdeI and EcoRI and cloned into vector pET15b (Novagen-Merck4Biosciences, Darmstadt, Germany). Recombinant CrHSK1 protein was overexpressed in E. coli BL21 (DE3) (Studier et al., 1990), and the protein with an N-terminally fused His6-tag was affinity purified on a NiNTA column as described (Maheswaran et al., 2004).

WESTERN-BLOT ANALYSIS

The concentration of purified recombinant CrHSK1 was determined by staining with amido black, using BSA as a standard (Popov et al., 1975). After separation of the proteins by SDS-PAGE on a 12% polyacrylamide gel, they were transferred to nitrocellulose membranes (Protran, Whatman) by a semidy blotting (Trans-blot SD, BioRad). Blots were blocked in 5% non-fat dry milk in Tris-buffered saline solution with 0.1% Tween 20 prior to an incubation of 1 h in the presence of anti-monoclonal anti-polyHistidine-peroxidase conjugate (A7058, Sigma-Aldrich). The dilutions of antibodies used were 1:2,000. The peroxidase activity was detected by an enhanced chemiluminescence assay (Promega).

GEL FILTRATION ANALYSIS OF OLIGOMERIC STATE OF CrHSK1

Gel-filtration experiments were performed using an ÄKTA micro chromatography system (GE Healthcare Life Sciences) operated at room temperature. A precision column GE Healthcare UK Ltd: Superose 6 Increase 3.2/300 was used with a running buffer consisting of 100 mM Tris pH 8.0, 40 mM KC1, 1 mM DTT, 20 mM MgCl2, 0.02% NaN3, CrHSK1 (50 µl of 1.5 µg/µl) was applied to the sample loop and chromatographed at a flow rate of 0.05 ml/min. The elution profile of the protein sample was recorded by UV detection at 280 nm and analyzed using UniCorn5 control Software (GE Healthcare). The GE Healthcare UK Ltd: Superose 6 column Increase 3.2/300 was calibrated from a standard plot of Kav versus molecular mass for ribonuclease (13.7 kDa), carbonic anhydrase (29 kDa), conalbumin (75 kDa), aldolase (158 kDa), and ferritin (440 kDa).

ENZYME ASSAY

A coupled enzyme assay was used to determine CrHSK activity in which the production of ADP was coupled to the oxidation of NADH by pyruvate kinase and lactate dehydrogenase as described previously (Minaeva et al., 2015). The reaction mix consisted of 100 mM Tris HCl pH 8.5, 50 mM KCl, 20 mM MgCl2, 0.4 mM NADH, 1 mM phosphoenolpyruvate, 2 mM ATP, 0.5 mM DTT, 11 U lactate dehydrogenase, 15 U pyruvate kinase, and 0.25 mM homoserine (HoS). The reaction was started by adding 2 µg HSK. The reaction was recorded over 10 min with a SPECCORD 210 photometer (Analytik Jena) at 340 nm. Phosphorylation of one molecule of HOS is proportional to the oxidation of one molecule of NADH, as indicated by a linear decrease in absorbance at 340 nm. One unit of HSK catalyzes the conversion of 1 µmol of HoS min−1, calculated with the molar absorption coefficient of NADH (ε340 = 6178 L mol−1 cm−1). Means of triplicate experimental determinations are shown with a standard deviation < 5%. The enzymatic parameters Km, kcat, Hill slope, and IC50 were calculated from the velocity slopes using the GraphPad Prism-6.01 software program (GraphPad Software, USA).

SYNTHESIS OF CDNA AND QUANTITATIVE REAL-TIME PCR (qRT-PCR) ANALYSIS

For experiments, cells grown at 22 °C to log phase (1-2 106 cells/ml) were collected by centrifugation, resuspended in TAP, and where indicated, freshly aliquoted H2O2 was added to cultures to a final concentration of 1 mM. RNA samples were prepared from cells collected at 0.5, 1, 1.5, 2 or 3 h after a H2O2 treatment. Total RNA was isolated as described previously (Ermilova et al., 2010). The quality of the RNA preparations was estimated by agarose gel electrophoresis, and RNA concentration and purity were determined spectrophotometrically (SmartSpec Plus, Bio-Rad). RNA (1 µg) was treated with RNase-Free DNase I (Fermentas) in 10 µl at 37 °C for 15 min. The reaction was stopped with 0.43 µl of 50 mM EDTA at 80 °C for 10 min.

Reverse transcription was performed with RevertAid H Minus First Strand cDNA Synthesis Kit according to the manufacturer’s instructions (Thermo Scientific). Primers for RT-qPCRs were chosen based on ≥ 90% primer efficiency and on a single melt curve. Each reaction contained the master mix, 5% DMSO, 200 nM of each primer, and cDNA corresponding to 10 ng input RNA in the reverse transcriptase reaction. Real time qPCR was performed on the Light Cycler Instrument (CFX96 Real-Time PCR Detection System, Bio-Rad) using
SYBR Green I as a fluorescent dye. The reaction conditions were as follows: 95 °C for 3 min, followed by cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s, up to a total of 40 cycles. All reactions were performed in triplicate with at least two biological replicates. Controls without template or reverse transcriptase were included. The threshold cycle (Ct), defined as the fractional cycle at which the fluorescence signal becomes significantly different (Ct), defined as the fractional cycle at which the transcriptase were included. The threshold cycle replicates. Controls without template or reverse performed in triplicate with at least two biological conditions were as follows: 95 °C for 3 min, followed SYBR Green I as a fluorescent dye. The reaction

results

characterization of Chlamydomonas HSK protein

The predicted full-length HSK1 polypeptide (A8I826 CHLRE) encoded by the C. reinhardtii HSK1 cDNA is 358 aa with a calculated molecular weight of 3629 Da. Representative plant and bacterial HSK sequences were aligned with the Chlamydomonas HSK sequence (Fig. 2). Two domain sequences are evident from amino acids 126 to 191 (GHMP kinases_N) and from 258 to 336 (GHMP kinases_C), which identify CrHSK as a member of the family of the GHMP superfamily (Zhou et al., 2000). Extremely high local identities occur over the signature pattern [LIVM]-[PK]-×-[GSTA]-×(0,1)-G-[LM]-[GS]-S-S-[GSA]-[GSTAC] that has been defined at the PROSITE (PS00627) (Fig. 2). This conserved sequence motif located in GHMP kinases_N domain of CrHSK from amino acids 132 to 143 is a putative ATP-binding domain (Bork et al., 1993 Tsay and Robinson, 1991). In addition, conserved are His-182 and Arg-283, both of which are functionally important residues of the homoserine binding site (Huo and Viola, 1996).

The phylogenetic tree of the HSKs presented in Fig. 3 suggests that the CrHSK is more closely related to HSK from another unicellular green alga, Chlorella variabilis. As expected, CrHSK is homologous to the HSKs from higher plants. The sequences from unicellular green algae form a tight cluster that groups together with clusters of sequences from higher plants, whereas sequences from heterotrophic bacteria, cyanobacteria and yeast build individual clusters only connected to Chlamydomonas sequence.

Subcellular localization was deduced from the analysis of the protein N-terminal sequence by several prediction programs (Predotar, TargetP, SignalP, and PSORT available at http://www.expasy.ch). Several prediction programs suggested that Chlamydomonas HSK might be targeted to chloroplasts or mitochondria. However, CrHSK is
Fig. 2. Comparison of the deduced amino acid sequences of *Chlamydomonas reinhardtii*, plant and bacterial HSK polypeptides. Aligned are deduced HSK sequences from *Chlamydomonas reinhardtii* (Cr; GenBank accession number EDP06874), *Arabidopsis thaliana* (At; AAD33097), *Oryza sativa japonica* (Os; XP_015624618.1), *Solanum lycopersicum* (Sl; XP_004236876.1), *Synechococcus elongatus* PCC 7942 (Se; ABB57470.1), *Synechocystis* sp. PCC 6803 (Sc; BAA17691.1), *Escherichia coli* (Ec; EDV66193.1), *Bacillus subtilis* (Bc; AEP92239.1). Residues highlighted in black are identical or conserved in at least 60% of all aligned HSK proteins. Amino acids in a gray background represent similar residues. Upper-lines I and II refer to two domain sequences of the GHMP superfamily, GHMP_kinases_N and GHMP_kinases_C, respectively. Alignments were made with the ClustalW program and refined manually.
homologous to the HSK from *Arabidopsis* (Fig. 3), which is shown to be localized in the chloroplast. We predicted *Chlamydomonas* HSK as chloroplast protein based on homology to *Arabidopsis* (Gen Bank AAD33097). Using the ChloroP algorithm (Emanuelsson et al., 1999, 2007), the HSK protein of *Chlamydomonas* is predicted to be cleaved after amino acid 30. The calculated molecular weight of predicted mature CrHSK polypeptide is 32874 Da. The size of the recombinant His6-tagged CrHSK
Fig. 4. Characterization of *Chlamydomonas* recombinant HSK1. 1 - The size standards; 2- SDS-PAGE gel electrophoresis (12%) of the purified His$_6$CrHSK1-tp. 3- the immunoblotting of purified His$_6$CrHSK1-tp using His-tag antibodies. Each lane was loaded with 10 µg protein. The position of molecular weight markers is shown at the left side.

lacking the transit peptide sequence His$_6$CrHSK-tp (calculated Mol weight 33927 Da) (Fig. 4) indicate that the prediction of the transit peptide is probably correct.

The mature CrHSK was only 28% identical to *E. coli* ThrB, 21% identical to *Bacillus subtilis* ThrB, 34% identical to *Synechococcus* PCC 7942 ThrB and 32% identical to *Synechocystis* sp. PCC 6803 ThrB. At the same time, the deduced amino acid sequence of CrHSK shares high overall identity with other representative HSKs from higher plants including: *Arabidopsis thaliana* (56.8%), *Oryza sativa japonica* (55.7%) and *Solanum lycopersicum* (58.8 %). These data are consistent with the phylogenetic analysis (Fig. 3).

**OLIGOMERIC STRUCTURE OF CrHSK1**

HSK proteins are usually dimeric proteins. To experimentally determine whether CrHSK1 forms a homodimer as well, the recombinant CrHSK protein was purified to near electrophoretic homogeneity (Fig. 4). His$_6$-tagged CrHSK-tp chromatographed as a 62.71 kDa protein during gel filtration whereas under denaturating conditions, it migrated as a 34 kDa band on SDS-PAGE implying that, like other HSKs, CrHSK1 is a homodimer (Fig. 5).

**KINETIC ANALYSIS OF THE HSK PROTEIN**

An assay measuring the formation of ADP (product of the HSK reaction) by coupling it to the oxidation of NADH was used to characterize the kinetic properties of the HSK protein. The activity of CrHSK in the coupled assay system resulted in a continuous and linear oxidation of NADH until consumption of NADH. The reaction velocity was directly proportional to the amount of enzyme added.

The activities of CrHSK were measured at various pH and temperature conditions. As in higher plants (Aarnes, 1976; Baum et al., 1983),
The Chlamydomonas enzyme had a pH optimum between pH 8.5 and 9.0 (Fig. 6, a). It showed high activity up to 50 °C with a peak at 37 °C which then decreased at higher temperatures (Fig. 6, b). Moreover, the CrHSK was stimulated by KC1, and maximal activity was attained between 30 and 50 mM (Fig. 6, c). The CrHSK protein also required Mg2+, since the enzyme did not exhibit any activity in the absence of Mg2+ (Fig. 6, d). The Chlamydomonas enzyme showed apparent Km value of 0.2 mM for ATP Reaction conditions were chosen at the optimal pH conditions and near saturating ATP, K and Mg concentrations.

As suggested, L-homoserine was used as substrate for CrHSK (Fig. 6, e). The apparent Km value determined for L-homoserine was 0.107 mM. As the CrHSK can be feedback regulated, we tested the effects of L-Cys, L-Ile, L-Thr, L-Met, L-Lys, and S-adenosyl-L-methionine on the activity of the purified enzyme. There was no significant inhibition of the CrHSK by these metabolites up to 10 mM (data not shown), with the exception of 10 mM Ile, which decreased CrHSK activity by 13% (Fig. 6, f, insert).

Expression of CrHSK1 is induced by H2O2

Since HSK1 in Chlamydomonas catalyzes an important step in the biosynthesis of methionine (Fig. 1), which is highly sensitive to oxidative stress, it was interesting to know whether or not CrHSK1 transcription is affected by H2O2. Treatment of Chlamydomonas cells with 1 mM H2O2 resulted in a 1.7-2-fold induction of this gene over the 1-1.5-h exposure period. During further exposure to peroxide, CrHSK1 transcript level decreased back to the control level. The H2O2-induced increase in HSP70A and MSD3 (encoding plastid-localized superoxide dismutase MnSOD3) transcripts abundance was used as a positive control to demonstrate proper responses in wild-type cells (Shao et al., 2008). The transient nature of CrHSK mRNA accumulation in response to exogenous hydrogen peroxide is similar to that seen with other H2O2-induced transcripts (Fig. 7; Blaby et al., 2015).

Discussion

Homoserine has been considered a precursor for threonine in higher plants (Azevedo et al., 1997; Jander and Joshi, 2009), but no information is available on the enzyme from photosynthetic eukaryotic microorganisms. In this work, HSK1 from Chlamydomonas was characterized with respect to its regulation and expression patterns.

The PROSITE N-terminal signature [LIVM]-[PK]-×-[GSTA]-×-[GSA]-×-[LM]-[GS]-S-S-[GSA]-[GSTAC], common to all prokaryotic HSKs is extremely well conserved within higher plants and Chlamydomonas (Fig. 2). This motif is seen in GHMP_kinases N domain and takes part in the binding of ATP. The phylogenetic tree of the HSKs presented in Fig. 3 indicates a stronger homology of CrHSK with the higher plants HSK proteins than with other HSKs from bacteria or yeast. Although nuclear-encoded, the HSK proteins are located in the chloroplasts of higher plants (Lee and Leustek, 1999; Lee et al., 2005). From the analysis of the localizations demonstrated for homologous proteins in higher plants Chlamydomonas HSK is predicted as chloroplast protein. The N-terminal part of CrHSK1 until residue A31 is predicted to encode a chloroplast transit peptide that is cleaved in the mature protein (ChloroP algorithm, Emanuelsson et al., 1999, 2007). The predicted molecular weight of mature CrHSK1 is consistent with data obtained for recombinant protein (Fig. 4). As expected, CrHSK1 forms a dimer (Fig. 5).

The maximum enzyme activity is observed at pH 8.5-9.0 and at 37 °C (Fig. 6, a-b). This enzyme has an apparent Km for L-homoserine of 0.107 mM and for ATP of 0.2 mM and it requires K+ and Mg2+ for activity (Fig. 6 c-e). In contrast to bacterial ThrB proteins (Shames and Wedler, 1984), CrHSK1 is not inhibited by homoserine concentrations above its optimum (Fig. 7, f). This difference is not surprising considering the low degree of identity between CrHSK1 and bacterial ThrB. On the other hand, Chlamydomonas HSK1 is nearly identical to the higher plant enzymes in molecular weight and substrate Km values (Lee et al., 2005). In addition, like plant enzymes (Riesmeier et al., 1993; Lee and Leustek, 1999), CrHSK1 has lost control by Asp-pathway amino acids and SAM. Although CrHSK showed a decrease in activity with Ile (Fig. 6, f), the non-physiologically high levels (10 mM) required for inhibition suggests that Ile probably does not play a role in regulating the enzyme in vivo. Prokaryotic homoserine kinases are involved only in production of Thr and Ile (Greene, 1996; Patte, 1996), while Chlamydomonas and higher plant enzymes have additional functions in the biosynthesis of methionine. The differences between
Fig. 6. Kinetic properties of the CrHSK1. a - The effect of pH on the activity of enzyme; b - the effect of temperature on the activity of enzyme; c - the effect of KCl on the activity of enzyme; d - the effect of MgCl₂ on the activity of enzyme; e - the effect of ATP on the activity of enzyme; f - the effects of HoS (black line) and 5 mM Ile (dotted line) on the activity of enzyme. Inset shows the effect of increasing concentrations of Ile on CrHSK1 activity. Assays were performed as indicated in Materials and Methods. The curves were fitted with GraphPad prism software. Standard deviations from triplicate experiments are indicated by error bars. HoS - homoserine, Ile - isoleucine.
prokaryotic and plant homoserine kinases might be a result of the differences in functions.

Considerable attention has been paid to the potential importance of transcriptional regulation of CrHSK1. Dark-light shift, nitrogen depletion, hypoxia, heat and cold stresses resulted in decreased CrHSK1 mRNA levels (data not shown). Here we found that CrHSK1 mRNA levels are increased in cells subjected to exogenous H₂O₂ (Fig. 7). Our data suggest that the CrHSK might make a contribution to oxidative stress resistance. However, the reason of why CrHSK1 transcript levels are induced in response to peroxide treatment is still an enigma. The generation and characterization of the CrHSK knock-down strains is now required to help understanding the physiological role of this enzyme in oxidative stress response.

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Author contributions
Tatiana Lapina: performed the experiments; Karl Forchhammer: wrote the paper; Elena Ermilova: original concept; analysis of data; wrote the paper.

References


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