Cellular Amino Acid Concentrations and Regulation of Aspartate Kinase in the Thermophilic Phototrophic Prokaryote *Chloroflexus aurantiacus*

Jobst-Heinrich Klemme, Gisela Laakmann-Ditges, and Jutta Mertschuweit

Institut für Mikrobiologie und Biotechnologie der Universität Bonn, Meckenheimer Allee 168, D-5300 Bonn 1, Bundesrepublik Deutschland

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Aspartate kinase (AK, EC 2.7.2.4) from the thermophilic, phototrophic prokaryote, *Chloroflexus aurantiacus,* was partially purified and separated from homoserine dehydrogenase (HSDH, EC 1.1.1.3). The molecular weights as determined by gel filtration were 130,000 and 46,000, respectively. HSDH had a moderately high thermal stability (50% inactivation at 84 °C) and displayed its activity optimum at 72 °C. By contrast, AK had its activity optimum at 52 °C (with a break-point in the Arrhenius plot at 42 °C) and was much less thermostable (50% inactivation at 67 °C). The K_m-values for aspartate and ATP (determined in a pyruvate kinase-coupled test system) were 10.5 and 0.63 mM, respectively. The enzyme was strongly inhibited by L-threonine (K_i = 10 μM) and activated by alanine, isoleucine, valine and methionine. L-Threonine acted as a mixed-type inhibitor in respect to aspartate, and non-competitively in respect to ATP. Contrary to AKs from Rhodospirillaceae, the enzyme from *Chloroflexus aurantiacus* was not subject to a concerted feedback inhibition by two amino acids of the aspartate family. The regulatory properties of the aspartate kinase are discussed in relation to the cellular amino acid concentrations.

Introduction

Chloroflexus aurantiacus is a filamentous, gliding, thermophilic phototrophic prokaryote occurring in hot springs of low sulfide content where it forms extended mats in syntrophic association with thermophilic cyanobacteria of the genus *Synechococcus* [1]. Although several strains of *Chl. aurantiacus* have successfully been grown under photoautotrophic conditions with sulfide as electron donor [2, 3], optimal growth of most laboratory cultures occurs only in nutritionally rich media containing yeast extract and/or peptone. Our experience with *Chl. aurantiacus* strain Ok-70-fl is that yeast extract can partly be replaced by casamino acids or a mixture of glutamate and the amino acids of the "aspartate" (met, lys, thr) and the "ilv" (ile, leu, val) group [4]. Such "conditional" requirement for certain amino acids may be caused by deficiencies of the related enzyme systems (enzymes of the "aspartate" and the "ilv" family). In previous papers [5], we have described the properties of two isozymes of L-threonine (L-serine) dehydratase from *Chl. aurantiacus* strain Ok-70-fl and have discussed their role in isoleucine biosynthesis. Here we report of the cellular amino acid concentrations in that strain and describe the regulatory properties of its aspartate kinase.

Abbreviations: ATCC, American Type Culture Collection; AK, aspartate kinase; HSDH, homoserine dehydrogenase; *Chl., Chloroflexus;* TCA, trichloroacetic acid.

Reprint requests to Prof. Dr. J.-H. Klemme.

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Materials and Methods

Chl. aurantiacus Ok-70-fl (ATCC 29365) was obtained from Dr. Karin Schmidt, Institut für Mikrobiologie, University of Göttingen. The organism was grown photosynthetically at 53 °C in a complex medium (if not otherwise noted) [6]. The cells were harvested after 4–6 days (when the specific activity of AK was at its maximum of about 40–60 nmol/min·mg protein), washed twice in 10 mM K-phosphate, pH 7.5, and resuspended in the same buffer to a concentration of about 0.4 g (wet weight) per ml. Cell suspensions could be stored at −18 °C for several months without loss of AK and HSDH activity.

Before rupture of cells by ultrasonic treatment, the suspension was supplemented with 30 μM L-threonine as a stabilizing effector of AK. The homogenates were freed of intact cells and cell debris by 15,000 × *g* centrifugation (4 °C, 30 min) and then subjected to ultracentrifugation (4 °C,

130,000 × *g*, 120 min) yielding 3 clearly separated cell fractions: a deep green sediment, a yellowish intermediate supernatant, and an orange-colored top layer. Activities of AK and HSDS were contained in the yellowish intermediate supernatant.

The enzymes were purified by the following steps. The supernatant from the ultracentrifugation step was treated with calcium phosphate gel prepared according to [7]. In the first step, 1.46 g gel/g protein was added. The gel was removed by centrifugation and discarded. Then, the pH of the enzyme solution was adjusted to 6.5 with 1 N HCl, and a second portion of gel (1.24 g/g protein) was added. The gel was collected by centrifugation and the enzymes were eluted by 5 washings with small volumes of 0.5 M K-phosphate, pH 7.5. The combined washing fluids were concentrated by ultrafiltration (Ultrafiltration cell GN 10–50 of Berghof GmbH, Eningen, with BM-100 membranes) and then filtered through a column of Sephacryl S 300 (1.5 × 85 cm) equilibrated with 50 mM K-phosphate, pH 7.5, supplemented with 30 µM L-threonine as stabilizing effector of AK. This purification step resulted in the separation of HSDH and AK (Fig. 1).

HSDH activity was measured spectrophotometrically (334 nm) at 52 °C) in a reaction mixture with L-homoserine and NADP [8]. AK activity was measured at 52 °C either colorimetrically [9] or spectrophotometrically (334 nm) in a reaction system coupled to pyruvate kinase and NADH [10]. Enzyme activities are expressed as µmol/min (= units). 1 unit (U) is equivalent to 16.67 nkat. Protein concentrations in the enzyme preparations were determined by the method of Bradford [11]. For electrophoretic separations in polyacrylamide gels the system No. 1 a [12] was used. HSDH activity bands of gels were stained for 30 min at 50 °C in reaction mixtures containing 100 mM Tris-HCl, pH 7.5; 5 mM L-homoserine; 0.2 mM NADP; 50 µg phenazine methosulfate; and 2 mg nitroblue tetrazolium chloride. AK activity stains of gels were performed at 50 °C in reaction mixtures identical to those used in the colorimetric assay of the enzyme. Because of the instability of the color, the position of the activity band was marked by slightly cutting the gel.

Concentrations of cellular amino acids were determined by using an automated amino acid analyzer (Model LC 6000, Biotronik, München). For these experiments, cells from 1000 ml minimal medium (10 mM glucose as C-source, 5 mM $(NH_4)_2SO_4$ as N-source, ref. [13]) were frozen at −18 °C for 24 h, thawed and then extracted with 10% (w/v) trichloroacetic acid [14]. 200 µl of combined TCA extracts were directly used for amino acid analysis. Amino acids in the lithium citrate-eluate from the cation-exchange column were converted to the corresponding ninhydrin derivatives and then analyzed colorimetrically. Specific amino acid concentrations (µg/mg dry weight) were converted to mM by assuming that 1 mg cellular protein corresponds to 8 µl cytoplasm [15].

Enzymes, coenzymes, and marker proteins for molecular weight determinations were obtained from Boehringer, Mannheim; amino acids, bovine serum albumin, and chemicals for electrophoresis from Serva, Heidelberg; Sephacryl S 300 and Superose 6 from Pharmacia-LKB, Freiburg; and all other chemicals from E. Merck, Darmstadt.

Results

As a biosynthetic enzyme, aspartate kinase should be at its maximum level of expression in cells growing in a minimal medium. However, the highest specific activities of AK (0.04–0.06 U/mg

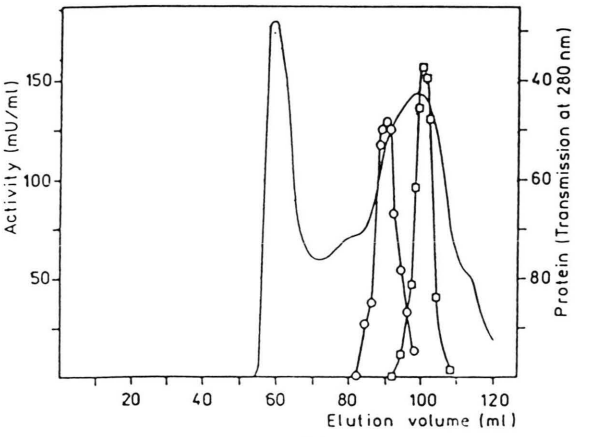

Fig. 1. Separation of aspartokinase and homoserine dehydrogenase in a cell extract of *Chl. aurantiacus* Ok-70-fl by gel filtration. 2 ml extract (eluate from Ca-phosphate gel; 8.4 mg protein) was filtered through a column (1.5 × 85 cm) of Sephacryl S 300 equilibrated with 50 mM K-phosphate, pH 7.5, supplemented with 30 µM L-threonine. Elution rate: 10 ml/h. Volume of fractions: 1 ml. ○: AK activity; □: HSDH activity; ——: protein. Specific activities of the peak fractions were 0.92 (AK) and 0.84 (HSDH) U/mg protein, respectively.

protein) were recorded in extracts from *Chl. aurantiacus* Ok-70-fl cells having been grown in media containing 0.5 to 1% (w/v) yeast extract. Extracts from cells grown in minimal medium had only very low AK activities (0.005–0.01 U/mg protein). Thus, the enzyme was purified from cells grown in complex media. The procedure outlined in the foregoing section yielded preparations with maximum specific activities of about 0.8–1.0 μmol/min·mg protein (peak fractions of the Sephacryl S300 eluate). The data of Fig. 1 show that the gel filtration step effectively separated AK and HSDH activities, *i.e. Chl. aurantiacus* Ok-70-fl does not contain a multifunctional AK/HSDH complex.

Gel filtration of partially purified AK and HSDH preparations through calibrated Superose 6 columns indicated molecular weights of 130,000 ± 6000 and 46,000 ± 3000, respectively. It should be noted that gel electrophoretic separation of proteins in cell extracts with subsequent activity staining always yielded only one separate band of AK and HSDH activity with R_f-values of about 0.23 (AK) and 0.54 (HSDH). The partially purified AK and HSDH preparations still contained up to 9 protein bands when analyzed by polyacrylamide gel electrophoresis.

The thermal stability of AK was relatively low. In the absence of effectors, 50% inactivation during a 10 min incubation occurred at 67 °C (84 °C for HSDH). The temperatures of maximum catalytic activity were 52 °C (AK) and 72 °C (HSDH).

Table I. Major properties of aspartate kinase from *Chl. aurantiacus* Ok-70-fl.

Mol. wt.*	130,000 ± 6000
Thermal stability (50% inactivation point)**	67 °C
Temperature optimum of activity	52 °C
Activation energies (kJ/mol)	61 (<42 °C) 17.3 (>42 °C)
Inhibitors***	thr, nor–leu, nor–val, pro
Activators***	ala, ile, val, met, leu, phe, tyr

* Determination by gel filtration through Superose 6.
** Incubation for 10 min at temperature tested.
*** Only L-forms considered; compounds arranged according to their effectiveness.

When plotted in an Arrhenius diagram (log v *vs.* T^{-1}), the HSDH kinetic data yielded a linear function up to the activity maximum at 72 °C (activation energy E_A = 49.9 kJ/mol), whereas the AK data gave a biphasic plot with a break point at 42 °C and activation energies of E_A = 61 kJ/mol (<42 °C) and E_A = 17.3 kJ/mol (>42 °C), respectively.

Besides L-threonine, also L-norleucine, L-norvaline, and L-proline inhibited the AK (99, 90, 70 and 55% inhibition, respectively, with 5 mM of effector in the standard colorimetric assay). The enzyme was activated by amino acids with non-polar side chains. L-Alanine, L-isoleucine, L-valine and L-methionine were the most effective compounds with activation factors of 2.3, 2.1, 2.0 and 1.8, respectively, at an effector concentration of 1 mM in the standard colorimetric assay. The major general properties of AK are summarized in Table I.

The kinetic constants of AK were determined in a series of spectrophotometric coupled tests conducted at the optimum temperature of 52 °C. At nearly saturating concentrations of the second substrate, both aspartate and ATP gave hyperbolic v *vs.* (S) plots with Hill coefficients of n_H = 1. The apparent K_m-values for K-aspartate and ATP were 10.5 and 0.63 mM, respectively. The inhibition of AK by L-threonine (the most powerful inhibitor) was of the mixed type with aspartate as the variable substrate, and non-competitive with respect to ATP. For both experimental series, a K_i-value of 0.01 mM was determined. Plots of v *vs.* (I) measured at various aspartate and ATP concentrations were sigmoidal with Hill coefficients n_H in the range of 1.4–1.6 (Table II).

As evident from the data in Table III, the enzyme from *Chl. aurantiacus* was not subject to a

Table II. Kinetic and regulatory constants of aspartate kinase from *Chl. aurantiacus* Ok-70-fl.

K_m (asp)		10.5 mM
K_m (ATP)		0.63 mM
K_i (thr)		0.01 mM
Hill coefficients of saturation curves	asp:	n_H = 1
	ATP:	n_H = 1
	thr:	n_H = 1.4 (asp = 10 mM)
	thr:	n_H = 1.6 (asp = 50 mM)

Table III. Effect of L-amino acids on aspartate kinase from *Chl. aurantiacus* Ok-70-fl.

Amino acid added	Relative activity [%]
None	100
thr (20 μM)	32
thr (30 μM)	9
lys (1 mM)	92
thr (20 μM) + lys (1 mM)	34
met (1 mM)	160
thr (30 μM) + met (1 mM)	18
ile (1 mM)	232
thr (30 μM) + ile (1 mM)	205

Reaction mixtures (1 ml) with 50 mM K-aspartate; 3.3 mM $MgSO_4$; 2.5 mM ATP; 0.7 mM NH_2OH; 10 mM Tris-HCl; and 0.9 mg protein of partially purified enzyme preparation; pH of reaction mixture: 8.0. Colorimetric test. 100% activity corresponds to 0.6 U/mg protein.

Table IV. Concentrations of soluble L-amino acids in *Chl. aurantiacus* Ok-70-fl.

Amino acid	μg/mg Cell protein		Approximate cellular conc. (mean value) [mM]
	Exp. No. 1	Exp. No. 2	
ala	0.49	0.76	0.88
arg	0.09	0.19	0.10
asp	0.13	0.07	0.09
glu	7.25	5.05	5.23
glu–NH_2	0.16	0.28	0.19
gly	0.04	0.09	0.12
his	0.05	0.03	0.03
ile	0.47	0.92	0.67
leu	0.04	0.10	0.07
lys	0.11	0.06	0.07
met	0.03	0.05	0.03
orn	0.28	0.20	0.23
phe	0.15	0.37	0.20
pro	0.03	0.06	0.05
ser	0.04	0.06	0.06
thr	0.16	0.06	0.12
tyr	0.06	0.18	0.08
val	0.14	0.08	0.12

Amino acid concentrations were determined in two experiments with different cell batches. The approximate cellular concentrations were calculated from the mean value of the two measurements by assuming that 1 mg cell protein corresponds to 8 μl cytoplasm [15].

concerted feedback inhibition by two amino acids of the "aspartate family", neither by thr + lys, nor thr + met. The activators isoleucine and valine, but not methionine (all at 1 mM), nearly completely abolished the inhibitory effect of 30 μM threonine. This rather complex interplay of activating and inhibiting amino acids led us to analyze the cellular amino acid concentrations in *Chl. aurantiacus* Ok-70-fl.

Table IV shows that in *Chl. aurantiacus* Ok-70-fl, like in many other bacteria, glutamate and alanine constitute the major part of the cellular amino acid pool. The cellular concentration of threonine is about 10 times higher than the corresponding K_i-value of the AK, whereas the aspartate concentration is about 100-fold lower than the K_m-value. From the other amino acids related to AK regulation, isoleucine deserves attention. In *Chl. aurantiacus* its concentration relative to glutamate (ile/glu = 0.13) is about ten times as high as in other phototrophic bacteria [16].

Discussion

Aspartate kinases isolated from bacterial species with only one functional enzyme are either regulated by a "concerted" or "multivalent" feedback inhibition by two amino acids of the "aspartate family" (thr + lys or thr + met) [17, 18] or a "compensatory" feedback inhibition where the inhibitory effect of one amino acid (thr) is compensated by a second one (ile or met) [19]. Note that enterobacteria and some *Bacillus* species contain up to three isozymes of AK with different catalytic and regulatory properties [20, 21]. Our data show that L-threonine is a very powerful allosteric inhibitor (K_i = 10 μM) of the AK of *Chl. aurantiacus* Ok-70-fl. Contrary to other thr-sensitive bacterial aspartate kinases, the *Chloroflexus* enzyme is not subject to a "concerted" inhibition by two amino acids of the "aspartate family". Instead, its activity seems to be controlled by an interplay of inhibition and activation. In addition to isoleucine and valine, other amino acids with non-polar side chains activate the enzyme. However, at concentrations corresponding to the cellular pools, only isoleucine is able to compensate the strong inhibitory effect of threonine. Comparison of the kinetic data with the cellular amino acid concentrations makes it likely that AK activity in *Chloroflexus* is controlled by the ratio (ile)/(thr). Yen and Gest [19] have postulated a similar regulatory pattern of AK in *Rhodopseudomonas palustris*. The overall capacity of AK activity in *Chl. aurantiacus* Ok-70-fl would be sufficient to supply the cells with the pre-

cursors of "aspartate family" amino acids. However, the very low activity of AK in cells grown in minimal medium, together with the low cellular aspartate concentration and the strong inhibition of AK by threonine, could offer an explanation for the deficiencies of biosynthesis of "aspartate family" and "ilv" amino acids.

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