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Bifunctional Protein in Carrot Contains Both Aspartokinase and Homoserine Dehydrogenase Activities[1]

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ABSTRACT

We have purified homoserine dehydrogenase to homogeneity and subjected polypeptide fragments derived from digests of the protein to amino acid sequencing. The amino acid sequence of homoserine dehydrogenase from carrot (*Daucus carota*) indicates that in carrot both aspartokinase and homoserine dehydrogenase activities reside on the same protein. Additional evidence that aspartokinase and homoserine dehydrogenase reside on a bifunctional protein is provided by coelution of activities during purification steps and by enzyme-specific gel staining techniques. Highly purified fractions containing aspartokinase activity were stained for aspartokinase activity, homoserine dehydrogenase activity, and protein. These gels confirmed that aspartokinase activity and homoserine dehydrogenase activity were present on the same protein. This arrangement of aspartokinase and homoserine dehydrogenase activities residing on the same protein is also found in *Escherichia coli*, which has two bifunctional enzymes, aspartokinase I-homoserine dehydrogenase I and aspartokinase II-homoserine dehydrogenase II. The amino acid sequence of the major form of homoserine dehydrogenase from carrot cell suspension cultures most closely resembles that of the *E. coli* ThrA gene product aspartokinase I-homoserine dehydrogenase I.

AK[2] (EC 2.7.2.4) catalyzes the phosphorylation of aspartate to β-aspartyl phosphate. It is the first enzyme of the pathway leading to the synthesis of the essential amino acids lysine, threonine, methionine, and isoleucine in bacteria and higher plants. In higher plants, there are commonly at least two forms of AK that are differentially feedback-inhibited by the end products lysine and threonine (6, 13–15, 21). HSDH (EC 1.1.1.3) catalyzes the reversible conversion of aspartate semialdehyde to homoserine and is at the branch point leading to threonine, methionine, and isoleucine synthesis.

Regulation of carrot AK and HSDH activities from roots and cell suspension cultures has been studied extensively (6, 13–15, 19–21). AK activity has been separated into two forms that are inhibited by either lysine or threonine. HSDH has been purified to apparent homogeneity and characterized (16). Carrot HSDH activity reversibly converts between a threonine-insensitive form in the presence of K^+ and a threonine-sensitive form in the presence of threonine that possesses distinctly different electrophoretic mobilitites on PAGE gels stained for enzymatic activity. Antibody to this HSDH has been examined for specificity and cross-reactivity with soybean and *Escherichia coli* (22).

The cloning and sequencing of plant genes encoding these enzymes has not been reported; however, there is extensive literature concerning these genes and the enzyme activities from bacteria. In *E. coli*, there are two bifunctional enzymes (5); the *thrA* gene encodes AKI-HSDHI activities and the *metL* gene encodes AKII-HSDHII. AKIII is encoded by *lysC* and does not contain HSDH activity. In *Bacillus subtilis* (1, 4), *Brevibacterium lactofermentum* (12), *Rhodospirillum rubrum* (8), and *Saccharomyces cerevisiae* (18), HSDH and AK activities reside on separate proteins encoded by separate genes.

In this paper, we provide evidence that there is a bifunctional protein on which AK and HSDH activities both reside in the protein isolated from carrot cell suspension cultures. The amino acid sequence of this bifunctional protein resembles AKI-HSDHI, the product of the *thrA* gene of *E. coli*.

MATERIALS AND METHODS

Plant Material and Enzyme Extraction

Cell suspension cultures of carrot (*Daucus carota* L. cv Danvers) were grown in flasks containing 200 mL of defined liquid medium as described previously (13). HSDH activity was purified to homogeneity as described (16). The proteins from polyacrylamide electrophoretic gels containing HSDH were blotted onto nitrocellulose paper and sent to Dr. William Lane, Harvard University, for amino acid sequencing. Sequence data were obtained from peptides separated by reversed-phase chromatography of digests of HSDH with trypsin after it was determined that the N-terminal amino acid was blocked.

AK was purified by a series of column chromatography steps. The cells were harvested after 5 d and disrupted in a nitrogen bomb as described for HSDH except that the threonine concentration was reduced to 1 mM and 1 mM lysine was added to the extraction buffer (16). The crude extract was concentrated by precipitation with 60% ammonium sulfate. Protein was resuspended in a minimal volume of 50 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM lysine, 1 mM threonine, and 20% glycerol and loaded onto

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[2] Abbreviations: AK, aspartokinase; HSDH, homoserine dehydrogenase.

the first gel filtration at 4°C through Biogel[3] A-0.5m (4.5 cm × 47 cm) equilibrated with the same buffer. Fractions containing AK activity were combined and concentrated by the addition of an equal volume of saturated ammonium sulfate for a second gel filtration at 4°C through Sephadex G-150 (2.5 cm × 39 cm) equilibrated with the above buffer with the addition of 0.2 M KCl. Those fractions with AK activity were combined and concentrated by the addition of an equal volume of saturated ammonium sulfate. Before ion exchange chromatography using HPLC at room temperature, the sample was dialyzed against 16 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 1 mM lysine, 1 mM threonine, and 20% glycerol. The dialyzed sample was loaded onto an Altex Spherogel TSK DEAE-5PW column (21.5 mm × 15 cm). AK activity was eluted over 65 min with a gradient of 0 to 0.5 M KCl in 20 mM Mes, pH 6, 5 mM 2-mercaptoethanol, 1 mM lysine, 1 mM threonine, and 20% glycerol. Each of the two peaks of activity was resolved at pH 6 and chromatographed separately in the subsequent two chromatography steps. Fractions containing activity of peak I or peak II were combined, diluted fourfold, and applied to an Altex Spherogel TSK DEAE-5PW column (7.5 mm × 7.5 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 5 mM 2-mercaptoethanol, 1 mM lysine, 1 mM threonine, and 20% glycerol. Fractions containing AK activity were combined and diluted fourfold and reapplied to the column, which was equilibrated with the same buffer containing 20 mM aspartate. Fractions with AK activity were concentrated by filtation through Centricon-30 filters (Amicon) and stored at −20°C.

Enzyme and Protein Assays and Stains

Aspartokinase activity was assayed by the hydroxamate method (2). The assay mixtures contained 100 mM Tris, pH 8.0, 50 mM hydroxamate-KOH, 50 mM L-aspartate, 40 mM ATP, and 20 mM Mg_2SO_4 and were incubated at 37°C for 60 min. The reaction was terminated by the addition of a solution of 0.37 M $FeCl_3$, 0.20 M TCA, and 0.72 M HCl. One unit of activity produced 1 μmol β-aspartylphosphate·h^{-1}. Zero time controls were included. Activity was visualized on polyacrylamide gels (Phast System by Pharmacia, Upsala, Sweden) by incubating gel slices at 37°C in 50 mM Tris-HCl, pH 7.5, 50 mM aspartate, 10 mM ATP, 50 mM $MgSO_4$, 1 mM DTT, 20% glycerol, 0.014 g·mL^{-1} $CaCl_2$, 0.04 mL·mL^{-1} 0.02% Alizarin red S (17, 19). To show specific aspartate kinase activity, duplicate gel strips were incubated without aspartate and also in aspartate with 10 mM lysine and/or 10 mM threonine.

HSDH activity was assayed in the direction of coenzyme reduction as described previously (16). A unit of HSDH activity has been defined as the amount of enzyme producing a change of absorbance at 340 nm of 0.001·min^{-1}. To compare units with AK, a unit of HSDH activity is defined as $9.6 \cdot 10^{-3}$ μmol·h^{-1}. Enzyme preparations were analyzed on 8 to 15% gradient polyacrylamide gels using a Phast gel electrophoresis system. HSDH activity was located on the gel as described (13) using 60 mM Tris-HCl (pH 9.0), 150 mM KCl, 30 mM NAD^+, 24 mM homoserine, 0.22 mM 2-mercaptoethanol, 0.15 mM EDTA, 0.266 mg·mL^{-1} nitro blue tetrazolium, and 0.025 mg·mL^{-1} phenazine methosulfate incubated at 37°C. Gels incubated in the staining mixture lacking homoserine were used as controls.

The thinness and rigid backing of the Phast gels facilitate sequential staining for activity. Because of the white precipitate product, the gel was first stained for AK activity and photographed. The gel was then stained for HSDH activity and photographed. The nitro blue tetrazolium imparts a high background to the gel, masking any previous AK staining.

Protein was visualized on gels using silver nitrate according to the manufacturer's instructions (Pharmacia). The gels could be silver stained for protein after activity staining, but residual nitro blue tetrazolium caused a very high background, making photography difficult.

Western Blot Analysis

The backing of the Phast gel was removed using a razor blade. The protein in the polyacrylamide gel was then electroblotted onto nitrocellulose in 25 mM Tris, 20 mM glycine, pH 8.3, at 25 to 35 V for 1 h at 4°C. Excess protein binding sites were blocked by incubating the filters in 1 × Tris-buffered saline, 1.0% dry milk, and 0.5% BSA for 1 h at room temperature. Nitrocellulose filters were incubated with primary antibody (anti-HSDH antiserum from mouse), secondary and tertiary antibody, and the alkaline phosphatase activity visualized as described (22).

RESULTS

Protein with AK activity was partially purified by gel filtration and anion exchange chromatography. The total protein and enzymatic activity at each purification step are listed in Table I. Two peaks of AK activity were eluted from the DEAE column at pH 6.0 (Fig. 1) and identified according to their position of elution. The first eluting peak (28–32 min), AKI, was inhibited by lysine and was relatively insensitive to threonine (Table II). The second peak (44–48 min), AKII, was inhibited by threonine and was relatively insensitive to lysine. These two peaks were kinetically different as well as having different feedback inhibition patterns. The $K_{m(asp)}$ for AKI was approximately 1 mM and for AKII was approximately 9 mM. Experimental conditions could affect which AK activities were observed. AK activity inhibited by lysine was not observed unless lysine was added to the homogenization buffer. If the pH of the sample was lowered to pH 6 before anion exchange, sensitivity to lysine was lost, although the total amount of AK activity remained unchanged. If AK activity with sensitivity to both lysine and threonine were applied to cation exchange medium, activity did not bind even at pH 5 and sensitivity to lysine was lost. The amount of activity recovered in the wash was comparable to that applied. Furthermore, the lysine-sensitive activity could not be eluted from the cation-exchange medium with KCl.

Both forms of AK had HSDH activity associated with them, although the ratio of AK:HSDH activity was not constant (Table II). This ratio did not correlate with the inhibition

[3] The mention of vendor or product does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over vendors of similar products not mentioned.

Table I. *Purification of Aspartokinase*

Sample[a]	Enzyme	Protein	Specific Activity[b]	Purification	Recovery
	units	*mg*		*-fold*	%
Crude	1590	2870	0.55		
Biogel A-0.5	1550	590	2.6	4.8	97
Sephadex G-150	1130	190	5.8	11	71
DEAE, pH 6.0					
Peak I	180	10	17	30	11
Peak II	58	4.3	14	24	3.6
DEAE, pH 7.5					
Peak I	81	4	20	36	5.1
Peak II	45	3	15	15	2.8
DEAE, pH 7.5, aspartate					
Peak I	4	0.13	31	56	0.25
Peak II	4	0.25	16	30	0.25

[a] Data from 10 preparations were averaged. [b] Expressed as units·g^{-1} protein.

pattern of the AK form of the enzyme. Even though the AK forms were different, all had HSDH activity that was sensitive to threonine. The HSDH and AK activities coeluted during all steps of the purification procedure (Fig. 2). No HSDH activity was detected for AKI after the anion exchange step at pH 6, but this activity could be measured after anion exchange at pH 7.5. In contrast to AKI, HSDH activity was detected with AKII after chromatography at pH 6.

In addition to the copurification of AK and HSDH activities, the activities comigrated on native PAGE. Both AKI and AKII and a mixture of I and II (AKIII) had a band of AK activity migrating to the same location on native PAGE. After staining for AK activity, the gel was scanned with a laser densitometer to locate bands. After staining for HSDH, the gel was again scanned and the location of the bands compared. According to the densitometer, there was a matching band with both stains (data not shown). Frequently, forms I and III had an additional faster-migrating band of AK activity appearing on gels, but HSDH activity appeared consistently with the slower-migrating band (Fig. 3). With form II, there was usually only one band of AK activity, but the HSDH activity appeared as a ladder extending above the AK band (Fig. 3). This ladder has been described as aggregates of HSDH (16). Lack of AK activity appearing with the HSDH activity ladder was thought to be due to the lack of sensitivity of the AK activity stain on gels. A very active sample of AK (AK44II), which has about 10 times the activity of most AK samples, gives a ladder of AK as well as HSDH activity on a gel (Fig. 3).

The cross-reactivity with forms of AK by antibody made to purified carrot HSDH (22) was investigated. Equal amounts of protein of each AK form were subjected to PAGE. Half of the gel was stained for HSDH activity and the other half was electroblotted onto nitrocellulose. Form I had about half the HSDH activity of form II and the sample that was a mixture of forms I and II; all had different AK activity. The nitrocellulose blot was incubated with antibody specific to carrot HSDH (22). Antibody bound to all three samples of AK (Fig. 4), indicating immunological similarity among the forms.

Further evidence that the two enzymatic activities reside on the same polypeptide is provided by amino acid sequence

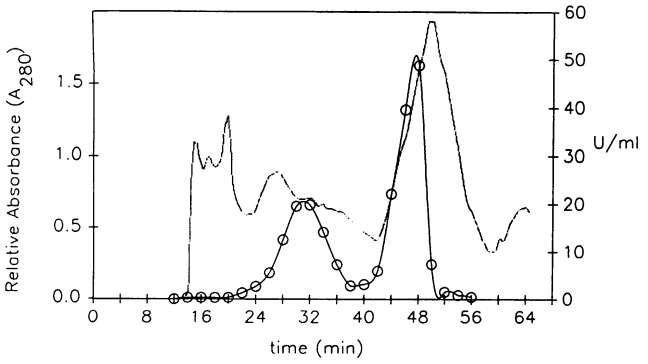

Figure 1. Resolution of two peaks of AK activity. The AK activity (O) in units·mL^{-1} of fractions eluted from the Altex Spherogel TSK-5PW DEAE (21.5 mm × 15 cm) column equilibrated with 20 mM Mes (pH 6), 5 mM 2-mercaptoethanol, 1 mM lysine, 1 mM threonine, and 20% glycerol is shown. The protein profile detected by a Waters Lambda-max monitor set to 280 nm is expressed as relative absorbance units.

Table II. *Inhibition Pattern of AK and Ratio of Dual Activities*

Prep Number	AK	HSDH	Ratio AK/HSDH	AK Activity +Lys[a]	AK Activity +Thr[a]
	units·mL⁻¹			*% control*	
ak32I	22	3.46	6.4	21	68
ak33I	8	21.1	0.38	39	71
ak33II	8	53.8	0.15	75	32
ak45II	48	30.7	1.6	91	33

[a] 10 mM.

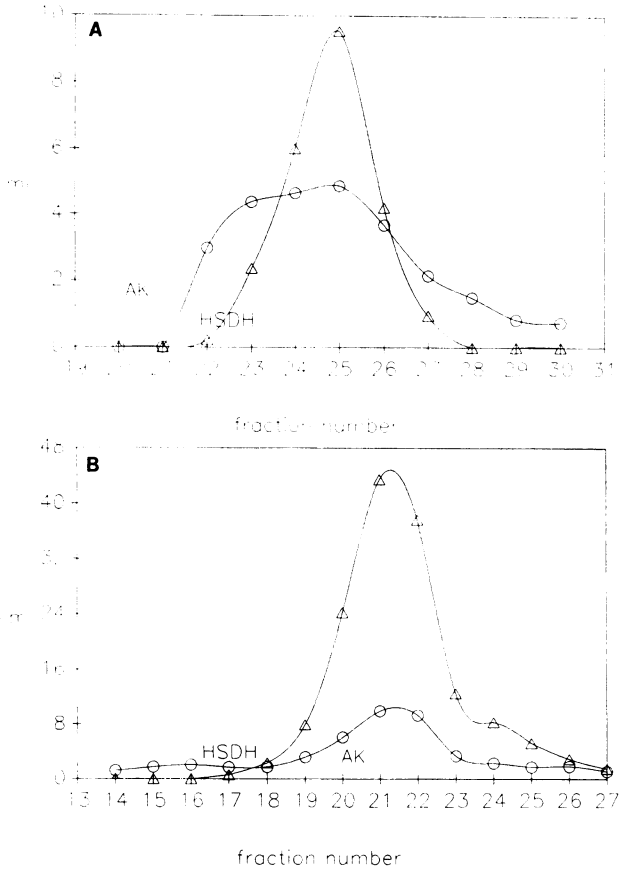

Figure 2. Coelution of AK and HSDH activities. Fractions collected from gel filtration and anion-exchange chromatography were assayed for AK and HSDH activities. A, Peak I activity from DEAE anion exchange at pH 7.5 with aspartate. B, Peak II activity from DEAE anion exchange at pH 6.

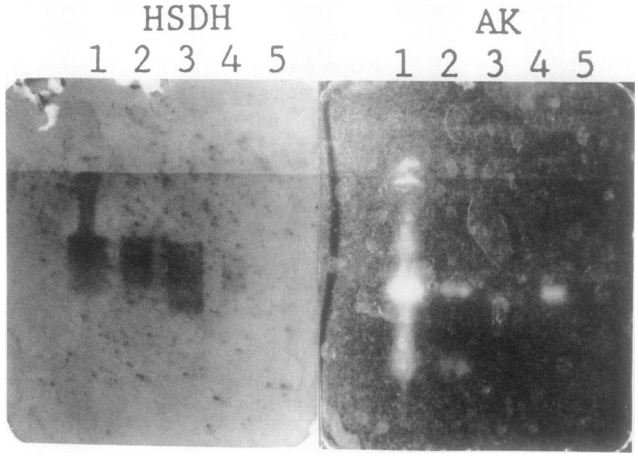

Figure 3. AK and HSDH activity stains on polyacrylamide gels. Samples (4 μL) of forms I and II were loaded onto 8 to 25% polyacrylamide gels. The gel was first stained for AK activity and photographed (right) and then stained for HSDH activity and photographed (left). Lane 1: ak44 with 1.2 units of AK (form II) and 127 units of HSDH; lane 2: ak41 with 0.08 units of AK (mix of forms I and II) and 7 units of HSDH; lane 3: akxx with 0.1 unit of AK (form II) and 7 units of HSDH; lane 4: ak32 with 0.09 unit of AK (form I) and 1 unit of HSDH; lane 5: ak31 with 0.09 unit of AK (mix of forms I and II) and 5.4 units of HSDH.

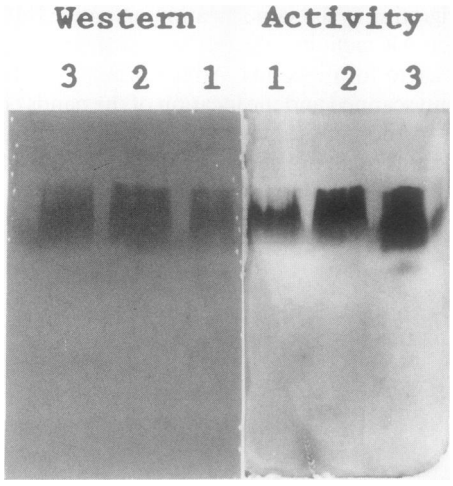

Figure 4. Western blot of samples of AK with antibody against HSDH. Equal amounts of protein (4 μg) of samples of AKI, II, and III were loaded onto 10 to 15% polyacrylamide gels. Half of the gel is electroblotted onto nitrocellulose for incubation with antibody and half is stained for HSDH activity. Lane 1: ak6 (form I) with 6 units HSDH; lane 2: ak33 (form II) with 22 units HSDH; lane 3: ak20 (mix of forms I and II) with 15 units HSDH.

data. Two peptide fragments derived from purified HSDH were sequenced. A sequence of 29 amino acids was obtained from peptide 97 (Table III). This sequence had some identity with portions of known HSDH genes from prokaryotes (5), especially with the HSDH regions of the two *E. coli* HSDHs (Table III). Carrot HSDH possessed 48% identity with the corresponding section of both AKI-HSDHI (ThrA) and 52% identity with AKII-HSDHII (MetL). The carrot HSDH possessed only 38% amino acid identity with HSDH from *B. subtilis* and 34% identity with *B. lactofermentum*.

A sequence of 21 amino acids was obtained from peptide 76. This sequence corresponded to a portion of the amino acid sequence of several known aspartokinases (Table IV) from prokaryotes and yeast (3, 4, 11, 18, 24). The amino acid identity between the carrot AK and the corresponding AK region of AKI-HSDHI (ThrA) of *E. coli* was 76% and identity with both AKIII (LysC) of *E. coli* and AK from *B. subtilis* was 52%. The identity with AKII-HSDHII (MetL) was 43% and was only 38% with yeast AK.

When the amino acid identities of the two sequences from carrot were compared with other known AK and HSDH sequences, carrot was found to be more closely related to the *E. coli* bifunctional AKI-HSDHI than to *E. coli* AKII-

Table III. *Sequence Identity of Peptide 97*

Source	Amino Acid Sequence	Identity	Ref.
		%	
Carrot	SYTHY FY-EAT VGAGL PIITT LQGLL ETGD		
ThrA (AKI-HSDHI)	SRRKF LK-DIN VGAGL PVIEN LQNLL NAGD	48	11
MetL (AKII-HSDHII)	TGRHW LY-NAT VGAGL PINHT VRDLI DSGD	52	24
B. subtilis	ENGCD TYFEAS VAGGI PILRT LEEGL SS-D	38	4
B. lactofermentum	-N-VD LYFEAA VAGAL PVVGP LRRSL -AGD	34	12

Table IV. *Sequence Identity of Peptide 76*

Source	Amino Acid Sequence	Identity	Ref.
		%	
Carrot	TLSYQ EAWEM SYFGA NVLHP R		
ThrA (AKI-HSDHI)	SMSYQ EAMEL SYFGA KVLHP R	76	11
MetL (AKII-HSDHII)	LLRLD EASEL ARLAA PVLHA R	43	24
LysC (AKIII)	EIAFA EAAFM ATFGA KVLHP A	52	3
B. subtilis	GISYD EMLEL ANLGA GVLHP R	52	4
Yeast	SVTPE EASEL TYYGS EVIHP F	38	18

HSDHII or to the *B. subtilis* AK and HSDH that are separate proteins. The identity of the carrot AK-HSDH with *E. coli* AKI-HSDHI was greater than the identity between any other two sequences; for example, the identity between *E. coli* AKI-HSDHI and *E. coli* AKII-HSDHII was only 34% for peptide 97 and 43% for peptide 76. The locations of the sequenced peptides from carrot have been identified on the AKI-HSDHI and are shown in Figure 5.

DISCUSSION

Enzymes involved in the synthesis of the aspartate family of amino acids appear to be relatively low in abundance. HSDH has been purified to homogeneity from maize (23) and carrot (16), whereas aspartokinase has been purified to homogeneity from maize (9) and partially purified from carrot (13, 14). There have been no indications in the literature that these two enzyme functions reside on the same protein in plants. Perhaps because there are AK activities sensitive to lysine and threonine but HSDH activity is sensitive only to threonine, a common polypeptide was not suspected. Although HSDH activity is associated with both lysine- and threonine-sensitive AK, the ratio of activities (Table II) is variable for reasons unknown at this time. In *E. coli* AKI-HSDHI, both enzymatic activities are inhibited by threonine. The *E. coli* AKIII is inhibited by lysine but it has no HSDH activity. In *E. coli* AKII-HSDHII, neither activity is inhibited by lysine or threonine. Because of these *E. coli* examples, an aspartokinase sensitive to lysine and associated with threonine-sensitive homoserine dehydrogenase appears to be inconsistent.

Other factors have prevented detection of these coincident activities. It had been observed that both AK and HSDH eluted off the gel filtration column at the same location, but it was assumed that it was a simple case of coelution of two large, similarly sized proteins. The purification protocol (16) for HSDH had already been established before the protocol for AK. In the protocol for purification of HSDH from carrot suspension culture cells (16), a heat denaturation step is utilized and activity of AK is lost after the heat denaturation step. Even though AK activity of the *E. coli* bifunctional AKI-HSDHI is lost after heating, the comparison was not made because a bifunctional protein in plants was not suspected.

Furthermore, in contrast to *E. coli,* not all bacteria have bifunctional AK-HSDHs. In *B. lactofermentum,* separate genes encode separate AK and HSDH proteins. The *B. subtilis* gene possesses two initiation sites to produce AKI and a truncated but functional AKII protein (4). In yeast, a gene encoding AK also has been identified (18); this gene does not appear to encode HSDH. HSDH has been extensively examined in *R. rubrum* (7, 8, 10), but there are no reports in the literature that this protein also contains AK activity.

The comparison of the amino acid sequences of carrot with the other known sequences suggests that the carrot AK-HSDH

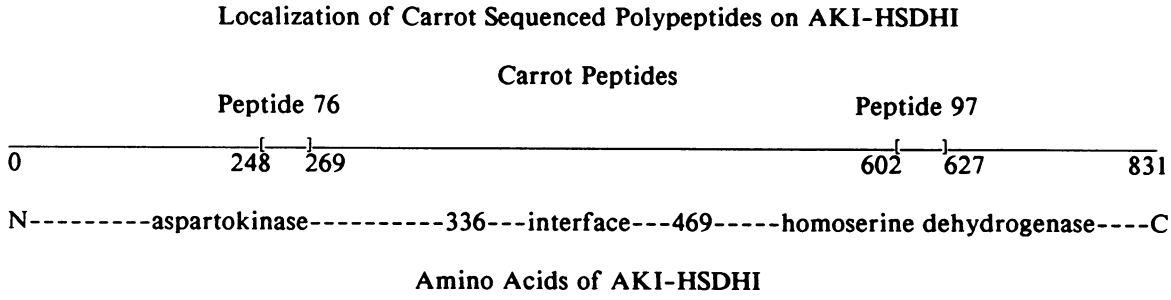

Figure 5. Localization of carrot sequenced polypeptides on *E. coli* AKI-HSDHI. The peptide sequences from Figures 3 and 4 are indicated on the homologous region of *E. coli* AKI-HSDHI. The regions of the *E. coli* protein with AK and HSDH activities are shown on either side of an interface region.

is most closely related to the AKI-HSDHI of *E. coli* because of the greater sequence homology at the AK portion of the protein. The carrot amino acid sequence has higher identity with the AKI-HSDHI than AKI-HSDHI has with the other *E. coli* fused protein AKII-HSDHII. Both carrot AK-HSDH and *E. coli* AKI-HSDHI are bifunctional enzymes with HSDH activities sensitive to threonine concentrations. The molecular mass of the subunit from carrot (85 kD) is similar to that of the subunits of *E. coli* AKI-HSDHI (86 kD) and AKII-HSDHII (88 kD). The sequence obtained from carrot is more like the sequences from the *E. coli* bifunctional sequences than the other known prokaryotic HSDHs. Although we have observed different AK activities in carrot, it is not clear if these are separate gene products as in *E. coli*. Besides the differences in inhibition to end products, the $K_{m(asp)}$ are also different. However, unlike *E. coli*, these different AKs in carrot may have a kinetically similar HSDH. Ligand binding is known to affect activity of the *E. coli* AKI-HSDHI. It has already been demonstrated that K^+ or threonine binding alters the carrot HSDH. The regulation by ligand binding of a protein with two enzymatic activities can be very complex. We are currently investigating if changing the lysine and/or threonine concentrations could cause a change in the inhibition pattern of AKI and AKII.

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