Reversal of Enzyme Regiospecificity with Alternative Substrates for Aspartokinase I from *Escherichia coli*[†]

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ABSTRACT: The substrate specificity of aspartokinase I has been examined by using both steady-state kinetic analyses and phosphorus-31 NMR spectroscopic studies. Analogues in which the α-amino group is either derivatized or replaced are not substrates or inhibitors for the enzyme, indicating the importance of the α-amino group as a binding determinant. The α-carboxyl group is not required for substrate recognition, and the α-amide or α-esters are competent alternative substrates. In addition, β-derivatized structural analogues, such as the β-hydroxamate, the β-amide, or β-esters, were found to be viable substrates. This was unexpected since the β-carboxyl group is the usual site of phosphorylation. The nature of the acyl phosphate products obtained from these β-derivatized alternative substrates has been characterized by coupled enzyme assays, oxygen-18-labeling studies, and phosphorus-31 NMR spectroscopy. These β-derivatized analogues are capable of productive binding to aspartokinase through a reversal of regiospecificity to make the α-carboxyl group available as a phosphoryl acceptor. Many, but not all, of these α-acyl phosphates have also been shown to be viable substrates for the next two enzyme-catalyzed steps in this metabolic pathway. This raises the possibility of producing enzyme-generated alternative substrates that can serve as antimetabolites for the downstream reactions in this biosynthetic pathway.

The aspartate biosynthetic pathway in *Escherichia coli* (Scheme I) uses L-aspartic acid as the precursor of the amino acids L-lysine, L-methionine, L-isoleucine, and L-threonine (Truffa-Bachi et al., 1974). The first and third reactions in this pathway are catalyzed by the bifunctional enzymes aspartokinase–homoserine dehydrogenase I and II. A third monofunctional enzyme, aspartokinase III, also catalyzes the phosphorylation of aspartic acid that is the commitment step in this biosynthetic pathway. These three enzymes are subject to differential regulation both by feedback inhibition and by repression at the gene level (Cohen, 1983). In *E. coli*, AK–HSD I[1] (EC 2.7.2.4) is a tetramer composed of identical subunits of molecular weight 86 000, the amino acid sequence of which has been determined (Falcoz-Kelly et al., 1972; Wampler, 1972). Each polypeptide chain contains the two catalytic activities; however, these activities reside on separate structural domains. The N-terminal moiety carries the kinase activity and the C-terminal region contains the dehydrogenase activity (Veron et al., 1972).

An active site histidine has been identified by modification with diethyl pyrocarbonate and protection by L-aspartic acid (Angeles et al., 1989). Activity loss was also observed upon derivatization of several tyrosine residues. The kinetic mechanisms of the reactions catalyzed by the two catalytic domains of this bifunctional enzyme have also been determined (Angeles & Viola, 1990).

The substrate specificity of AK–HSD I has been examined briefly by Shames et al. (1984). During an examination of the mode of action of antimetabolites with the enzymes of the aspartate–threonine pathway, it was observed that L-threo-3-hydroxyaspartic acid can act as an alternative metabolite to L-aspartic acid for the first four enzymes of the pathway, while 2-methylaspartic acid was a competent substrate of aspartokinase I, but the acyl phosphate product was not a substrate for ASA-DH, the next enzyme in the biosynthetic pathway. *N*-Methylaspartic acid exhibited no reactivity at all with aspartokinase I.

In this paper, we present a detailed study of the structural features required for substrate activity by aspartokinase I. The products of some of the unusual alternative substrates have been characterized by kinetic, isotope labeling, and NMR studies.

EXPERIMENTAL PROCEDURES

Materials. L-Aspartate α-benzyl ester, L-aspartate β-methyl ester, and L-aspartate diethyl ester were obtained from Chemical Dynamics Corporation. Oxygen-18-enriched water (97–98%) was purchased from Cambridge Isotope Laboratories. Purified samples of aspartate-β-semialdehyde and aspartate-β-semialdehyde dehydrogenase (ASA-DH) were provided by Dr. William E. Karsten. AK–HSD I was purifed from *E. coli* by the method of Karsten et al. (1985), suspended in 100 mM Tris-HCl, pH 7.5, containing 0.5 mM DTT, 0.1 mM EDTA, and 10% glycerol, and then stored in liquid nitrogen. Homoserine kinase was a generous gift from Dr. David E. Ash. All other substrates, substrate analogues, buffer salts, and coupling enzymes were purchased from Sigma.

Homoserine phosphate (HSP) was prepared enzymatically by following the procedure of Shames et al. (1984). The reaction mixture contained 0.15 M Hepes (pH 7.8), 0.25 M KCl, 10 mM $MgCl_2$, 0.15 M L-homoserine, 0.12 M ATP, and 40 units of homoserine kinase in a final volume of 15 mL.

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[1] Abbreviations: Aces, 2-[(carbamoylmethyl)amino]ethanesulfonic acid; AK–HSD I, aspartokinase–homoserine dehydrogenase I; ASA-DH, aspartate-β-semialdehyde dehydrogenase; DTT, dithiothreitol; Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; HSP, homoserine phosphate.

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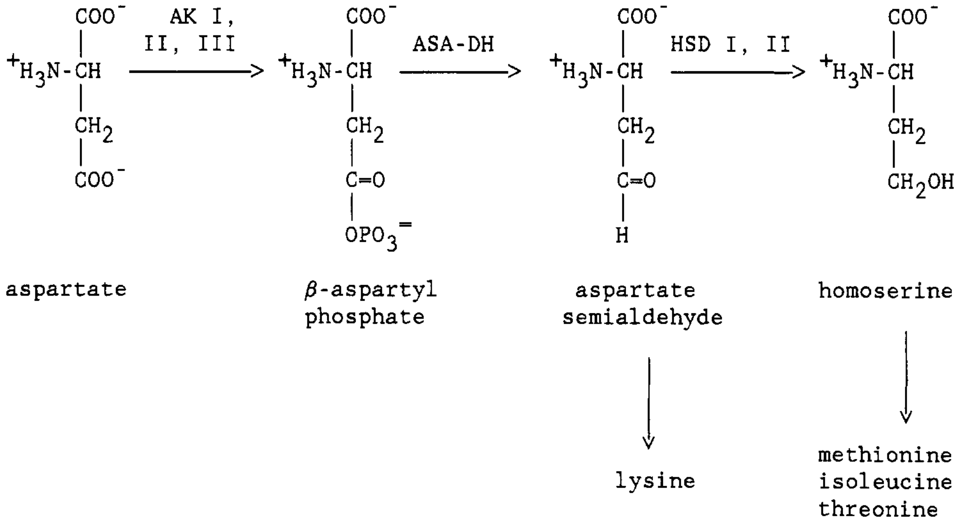
Scheme I: Aspartate Branch of Amino Acid Biosynthesis



After mild stirring at 37 °C for 12 h, the reaction mixture was evaporated to 5 mL at 40 °C and applied to a cation-exchange column (Dowex AG50W-X8; 200–400 mesh). The column was then eluted with deionized water. The fractions containing HSP were pooled, adjusted to pH 7.5, concentrated, and then stored at −20 °C. The product was characterized by ^{31}P NMR spectroscopy: $\delta = 0.21$ (t, $J = 6$ Hz). The concentration of the HSP solution was determined by incubating a known volume with alkaline phosphatase for 10–12 h in a solution containing 0.1 M glycine (pH 10.4), 1 mM $MgCl_2$, and 1 mM $ZnCl_2$. The incubation mixture was then assayed for inorganic phosphate by using the malachite green phosphate method (Lanzetta et al., 1979).

L-Aspartate β-benzyl ester was synthesized according to the procedure of Van Heeswijk et al. (1982), which involved formation of an L-aspartic acid–copper(II) complex of the *N,N,N′,N′*-tetramethylguanidinium salt and its subsequent alkylation. The free ester was isolated by precipitation and washing of the copper complex with acetone and treatment with an excess of EDTA (disodium salt) in water. The product was characterized by NMR spectroscopy. ^{1}H NMR δ: 7.3 (s, 5 H), 5.1 (s, 2 H), 3.9 (t, $J = 5$ Hz, 1 H), 2.7 (m, $J = 6$ Hz, 2 H). ^{13}C NMR δ: 176, 175, 138.4, 132.1, 131.6, 70.8, 54, 38.

Oxygen-18-labeled L-aspartic acid was prepared by a modification of the procedure of Khazaeli and Viola (1984). L-Aspartic acid (1.5 g) was dissolved in a 5 N HCl solution prepared by mixing 1 mL $H_2{}^{18}O$ (97–98%), 300 μL of DCl (20% in D_2O), 1 mL of 12 N HCl, and 620 μL of H_2O. The resulting solution was heated at 90 °C in a sealed 10-mm NMR tube. The extent of exchange was monitored by using the ^{18}O isotope shift on ^{13}C NMR. Carbon-13 NMR spectroscopy was conducted at 25 °C on a Varian VXR-300 NMR spectrometer operating at 75 MHz. Quantitative determination of the label was based on the integration of the ^{13}C NMR signals. The accumulation parameters for the proton-decoupled spectra were pulse angle, 43.5°; pulse delay, 10 s; and accumulation time, 1.5 s. Upon completion of exchange, the solution was adjusted to pH 3 and then cooled in an ice bath. The precipitate thus obtained was dried under vacuum overnight.

Enzyme Assays. Aspartokinase activity was routinely assayed in the biosynthetic direction by coupling the production of ADP with pyruvate kinase and lactate dehydrogenase. The 1.0-mL reaction mixture consisted of 100 mM Hepes(Tris), pH 8.0, 100 mM KCl, 5–10 mM magnesium chloride, 1.8 mM ATP, 0.7 mM phosphoenolpyruvate, 15 mM L-aspartic acid, and 8–15 μg of AK–HSD I. Initial velocities were measured at 30 ± 1 °C by following the disappearance of NADH at 340 nm by using a Perkin Elmer Lambda-1 spectrophotometer. In control experiments, the levels of the coupling enzymes were varied to ensure that the aspartokinase reaction was completely rate limiting, and no lags were observed in the disappearance of NADH upon initiation of the reaction by the addition of AK–HSD I.

An alternative method to measure the rate of aspartokinase reaction coupled the formation of β-aspartyl phosphate with ASA-DH. The reaction mixture consisted of 100 mM Hepes(Tris), pH 8.0, 100 mM KCl, 15 mM L-aspartic acid, 1.8 mM ATP, 5–10 mM $MgCl_2$, 0.1 mM NADPH, 3–15 μg of AK–HSD I, and 1–5 μg of ASA-DH. Initial velocities were measured by monitoring the oxidation of NADPH at 340 nm. Reciprocal plots at saturating amounts of the fixed substrate were fitted to the Michaelis–Menten equation to determine the kinetic parameters of the reaction.

Inhibition Studies. Analogues of L-aspartic acid and β-aspartyl phosphate were examined as inhibitors in the aspartokinase I reaction by using the coupled reaction with ASA-DH. The K_i (inhibition constant) was calculated from Dixon plots at fixed concentrations of L-aspartic acid (Segel, 1976). In some cases, a complete inhibition pattern was run in which both the inhibitor and L-aspartic acid concentrations were varied. These data were fitted to the equation for competitive inhibition to determine the inhibition constants. The kinetic data were analyzed by using BASIC versions of the Fortran programs of Cleland (1967).

Stability Studies on the Acyl Phosphate Products of the Aspartokinase I Reaction. An end point assay experiment to measure the stability of the acyl phosphates produced from the aspartokinase I reaction was conducted by incubating K_m levels of L-aspartic acid or its analogue in an assay mixture consisting of 100 mM Hepes(Tris), pH 8.0, 100 mM KCl, 3.6 mM $Mg(OAc)_2$, 1.8 mM ATP, and 7.5 μg of AK–HSD I at 30 °C. After 0.5–1 h of incubation, L-threonine (5 mM) was added to inhibit the enzyme. The rate of breakdown of the resulting acyl phosphates was determined by adding aliquots of this reaction mixture to an assay mixture containing 100 mM Hepes(Tris), pH 8.0, 0.1 mM DTT, 0.1 mM NADPH, 5 mM L-threonine, and 1.32 μg of ASA-DH. Quantitation of the acyl phosphate was determined from the decrease in

Table I: Substrate Specificity of Aspartokinase I

substrate[a]	R_1	R_2	K_m (mM)	% V_{max} ADP assay	% V_{max} acyl-P assay
L-aspartic acid	-OH	-OH	1	100	100
D-aspartic acid[b]	-OH	-OH	12	3	3
L-aspartic acid amide	$-NH_2$	-OH	50	17	15
L-aspartate α-benzyl ester	$-OCH_2C_6H_5$	-OH	4	13	17
L-asparagine	-OH	$-NH_2$	4	24	15
L-aspartate β-benzyl ester	-OH	$-OCH_2C_6H_5$	3	16	11
L-aspartate-β-hydroxamate	-OH	-NHOH	15	21	14
L-aspartate β-methyl ester	-OH	$-OCH_3$	20	62	65
L-cysteine sulfinate	-OH	$-SO_2H$[c]	14	3	0
2-methyl-DL-aspartate[d]	-OH	-OH	9	22	0

[a] Parent structure: $R_2C(=O)CH_2CH(NH_3^+)C(=O)R_1$. [b] Change in stereochemistry at carbon-2. [c] Replacing the 4-carboxyl group. [d] Hydrogen replaced by a methyl group at carbon-2.

absorbance of NADPH using an ϵ_{340} of 6.22×10^3 M^{-1} cm^{-1}. The effect of temperature on the stabilities of the acyl phosphate products was investigated following the procedure described above, except that the rate of breakdown was monitored at 0 °C.

^{31}Phosphorus NMR Assay for Substrate Activity with Aspartokinase I. (a) Detection of Acyl Phosphates. The NMR spectra were obtained on a VXR-300 NMR spectrometer operating at 121 MHz for ^{31}P resonance and equipped with a field-frequency lock on the deuterium resonance. All the experiments were performed at 6 °C for 12–24 h with variable preacquisition delays. Accumulation parameters used for assays were as follows: pulse angle, 45°; pulse delay, 0.48 s; acquisition time, 0.80 s. Each reaction mixture (0.70 mL) contained 100 mM Hepes(Tris), pH 8.0, 100 mM KCl, 20% D_2O, 30 mM MgATP, 15 mM phosphoenolpyruvate, 30 mM either L-aspartic acid or analogue, 30 units of pyruvate kinase, and 3–15 units of AK–HSD I. All of the analogues of L-aspartic acid were initially incubated with excess L-aspartase to remove any contaminating aspartate, passed through an Amicon microconcentrator, and later lyophilized. The purity of the compounds was determined both by ^1H and ^{13}C NMR spectroscopy and by thin-layer chromatography.

(b) Hydrolysis of Acyl Phosphates. Phosphorus NMR spectroscopy was carried out at 121 MHz in 5-mm NMR tubes. Samples contained 20% deuterium oxide as internal NMR lock. Chemical shifts for phosphorus-containing compounds were reported relative to 85% phosphoric acid as an external standard. Accumulation parameters used for assays at 25 °C were pulse angle, 45°; pulse delay, 0.480 s; and acquisition time, 0.798 s.

A representative assay contained 100 mM Hepes(Tris), 100 mM KCl, 6 mM MgATP, and 27 μg of AK–HSD I. The substrate concentrations were 15 mM for L-aspartic acid or [^{18}O]L-aspartic acid, 40 mM for L-aspartate β-methyl ester, and 50 mM for L-cysteine sulfinate. Oxygen-18-enriched H_2O was added to a final concentration of 50% except in the case where [^{18}O]L-aspartic acid was used. The hydrolysis of the putative acyl phosphates was monitored at pH 7 and pH 9. All samples were kept at 4 °C between NMR spectral analyses. Control solutions were prepared in the same manner as the assay solutions except for the absence of either substrate or enzyme.

RESULTS

Substrate Specificity of Aspartokinase I. A wide range of structural analogues of L-aspartic acid have been examined as possible alternative substrates for the reaction catalyzed by aspartokinase I. Of these analogues, compounds with a derivatized α-carboxyl group, such as L-aspartate α-benzyl ester and L-aspartic acid amide, were recognized as substrates of aspartokinase I by using the coupled assays both for ADP and for acyl phosphate production. Unexpectedly, analogues with a derivatized β-carboxyl group such as L-asparagine, L-aspartate β-methyl ester, L-aspartate β-benzyl ester, and L-aspartate-β-hydroxamate also appeared to yield products in both of these coupled assays. L-Cysteine sulfinate and 2-methyl-DL-aspartate appear to be substrates only in the assay for ADP production. The kinetic parameters have been determined for each putative alternative substrate and these values are compared to those of L-aspartic acid (Table I). AK–HSD I shows relatively high substrate affinity for L-aspartate α-benzyl ester, L-asparagine, L-aspartate β-benzyl ester, and 2-methylaspartate; however, the maximum velocities observed for these compounds fall in the range of 10–25% that of L-aspartic acid. The relative V/K values for all of these structural analogues are less than 10% that of the physiological substrate. None of the N-substituted analogues of L-aspartic acid that were examined showed reactivity toward aspartokinase I (Table II). Likewise, AK–HSD I showed no reactivity toward the corresponding D-isomers of the derivatives of L-aspartate, although D-aspartic acid itself appears to be a poor substrate for the enzyme. The compounds which were observed to be alternative substrates in the aspartokinase I reaction were each found to be free of significant L-aspartic acid contamination by thin-layer chromatography and by assay with the highly specific enzyme L-aspartase.

It was initially thought that AK–HSD I may possess an esterase activity or that the activities which were observed were a result of the nonenzymic hydrolysis of these esters and amides. Substrate activity was observed with L-aspartate diethyl ester, a substrate analogue in which both carboxyl groups are derivatized. However, further examination showed that this observed activity could be accounted for by contamination with the monoethyl ester. Both explanations for this range of alternative substrates were eliminated when L-aspartate dimethyl ester was tested and no substrate activity was observed.

The requirements for a phosphoryl donor were also investigated using structural analogues of ATP. With the exception of 2′-deoxy-ATP, all of the triphosphates tested, including CTP, GTP, UTP, and β,γ-methylene-ATP, were found to be unreactive toward aspartokinase I. At saturating amounts of L-aspartic acid, MgdATP was observed to have a K_m of 1.8 ± 0.2 mM (2-fold higher than the K_m for MgATP) and a V_{max} of the same magnitude as that of MgATP.

Stability Studies on Acyl Phosphates. It would be useful, for the characterization of the reactions catalyzed with these alternative substrates, to isolate and identify the products of these reactions. However, all of the acyl phosphate products of the aspartokinase I reaction are susceptible to hydrolysis at 30 °C. The corresponding acyl phosphate products derived from L-aspartate α-benzyl ester and L-aspartate β-methyl ester

Table II: Inhibitors and Nonsubstrates of Aspartokinase I

structural analogues[a]	R_1	R_2	R_3	K_i (mM)
	A. Aspartate Analogues[b]			
L-norvaline	$-CH_2CH_2CH_3$	$-COO^-$	-H	3.1 ± 0.6
2-aminobutyrate	$-CH_2CH_3$	$-COO^-$	-H	5 ± 1
L-alanosine	$-CH_2N(O^-)NO$	$-COO^-$	-H	7.5 ± 0.9
2,4-diaminobutyrate	$-CH_2CH_2NH_2$	$-COO^-$	-H	10 ± 1
D-aspartate α-benzyl ester[c]	$-CH_2COO^-$	-COOBz	-H	11 ± 3
D-aspartate-β-hydroxamate[c]	$-CH_2CONHOH$	$-COO^-$	-H	16 ± 6
2-aminomalonate	$-COO^-$	$-COO^-$	-H	23 ± 4
L-norleucine	$-CH_2CH_2CH_2CH_3$	$-COO^-$	-H	24 ± 3
L-glutamate γ-methyl ester	$-CH_2CH_2COOCH_3$	$-COO^-$	-H	24 ± 5
DL-isoserine	$-CH_2NH_2$	$-CH(OH)COO^-$	-H	30 ± 7
L-valine	$-CH(CH_3)_2$	$-COO^-$	-H	32 ± 4
L-glutamate	$-CH_2CH_2COO^-$	$-COO^-$	-H	38 ± 1
D-asparagine[c]	$-CH_2CONH_2$	$-COO^-$	-H	39 ± 14
3-aminoglutarate	$-CH_2COO^-$	$-CH_2COO^-$	-H	45 ± 8
L-aspartate dimethyl ester	$-CH_2COOCH_3$	$-COOCH_3$	-H	71 ± 18
L-alanine	-H	$-COO^-$	-H	99 ± 20
L-isoleucine	$-CH(CH_3)CH_2CH_3$	$-COO^-$	-H	-[d]
L-α-aminoadipic acid	$-(CH_2)_3COO^-$	$-COO^-$	-H	-[d]
N-acetyl-L-aspartate	$-CH_2COO^-$	$-COO^-$	$-COCH_3$	-[d]
N-chloroacetyl-L-aspartate	$-CH_2COO^-$	$-COO^-$	$-COCH_2Cl$	-[d]
N-formyl-L-aspartate	$-CH_2COO^-$	$-COO^-$	-COH	-[d]
	B. β-Aspartyl Phosphate Analogues[e]			
homoserine phosphate	$-(CH_2)_2OPO_3^{2-}$	$-COO^-$	-H	6 ± 1
DL-2-amino-5-phosphonovalerate	$-(CH_2)_3PO_3^{2-}$	$-COO^-$	-H	8.4 ± 0.9
O-phospho-L-serine	$-CH_2OPO_3^{2-}$	$-COO^-$	-H	12 ± 3
DL-2-amino-4-phosphonobutyrate	$-(CH_2)_2PO_3^{2-}$	$-COO^-$	-H	61 ± 3

[a] Parent structure: $R_1CH(NHR_3)R_2$. [b] Competitive inhibitors vs L-aspartic acid. The K_i values were determined by a fit of the data to eq 2. [c] Change in stereochemistry at carbon-2. [d] Noninhibitors of the aspartokinase-catalyzed reaction. [e] Dead end inhibitors vs L-aspartic acid. The K_i values were determined by a fit of the data to the equation for competitive inhibition.

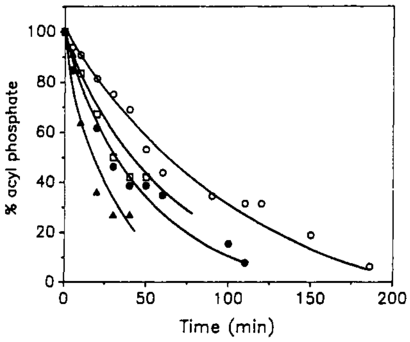


FIGURE 1: Nonenzymatic hydrolysis of the acyl phosphates generated by aspartokinase I from MgATP and (○) L-aspartic acid, (□) L-asparagine, (●) L-aspartate β-methyl ester, or (▲) L-aspartate-β-hydroxamate.

showed slightly greater stability than the phosphorylated products obtained from L-aspartate-β-hydroxamate, L-asparagine, and L-aspartic acid amide (Figure 1). However, all of these acyl phosphates, with half-lives ranging from 20 to 40 min, were less stable than L-β-aspartyl phosphate. Several attempts were made to trap these unstable products using a variety of reagents. In each case, interference from the higher levels of reactants precluded a definitive judgment on the identity of the products. Lowering the temperature of the incubation to 0 °C reduces the rates of hydrolysis of these phosphorylated compounds and allowed their direct detection by phosphorus-31 NMR spectroscopy.

^{31}Phosphorus NMR Studies. The time course of the aspartokinase I reaction was monitored by using phosphorus-31 NMR spectroscopy to detect the formation of the phosphorylated product of L-aspartic acid or its analogues. Using the natural substrate L-aspartic acid, the appearance of a peak corresponding to L-β-aspartyl phosphate ($\delta = -2.07$ ppm) was observed by using the standard reaction mixture at 6 °C. Subsequent hydrolysis of this acyl phosphate resulted in the accumulation of inorganic phosphate ($\delta = 2.41$ ppm). The assignments of the other peaks in the spectra were made on the basis of the known ^{31}P NMR spectra of ATP and ADP (Cohn & Hughes, 1960). Broadening of these signals is observed in the presence of Mg^{2+} ions. Quenching the reaction by adding EDTA eliminated the chemical exchange broadening, thus resulting in narrower signals.

When the reaction was first examined with the putative alternate substrates, no acyl phosphate peaks were detected. Peaks were observed corresponding to inorganic phosphate after the first hour of incubation of these analogues with the enzyme. A modified experimental setup was then employed to optimize the formation of the acyl phosphate products. Incorporation of phosphoenolpyruvate and pyruvate kinase in the reaction mixture allowed recycling of ADP to ATP to shift the equilibrium in the direction of acyl phosphate formation. After a 30-min incubation, a prominent resonance was observed for β-aspartyl phosphate which yielded L-aspartic acid and inorganic phosphate upon hydrolysis (Figure 2A). The formation of the alternate acyl phosphate products was then determined by separately incubating L-aspartate α-benzyl ester, L-aspartate β-methyl ester, L-asparagine, 2-methyl-DL-aspartate, and L-cysteine sulfinate with AK-HSD I. Spectra illustrating the production of acyl phosphates from β-derivatized aspartate analogues are shown in Figure 2B,C. The chemical shifts of the various phosphorylated products of these reaction mixtures are given in Table III. With the exception of the enzyme-generated acyl phosphate products, no hydrolysis of the other phosphorylated compounds was observed during the time course of the experiments. The instability of the various acyl phosphate derivatives was reflected by their low steady-state levels and the large P_i/acyl phosphate ratios in the NMR spectra. After completion of the NMR studies the reaction mixtures were examined by thin-layer chromatography. There was no indication of the presence of aspartic acid in the experiments in which the various α- or β-derivatized aspartates were examined. These results establish the stability of the various esters and amides under these reaction conditions

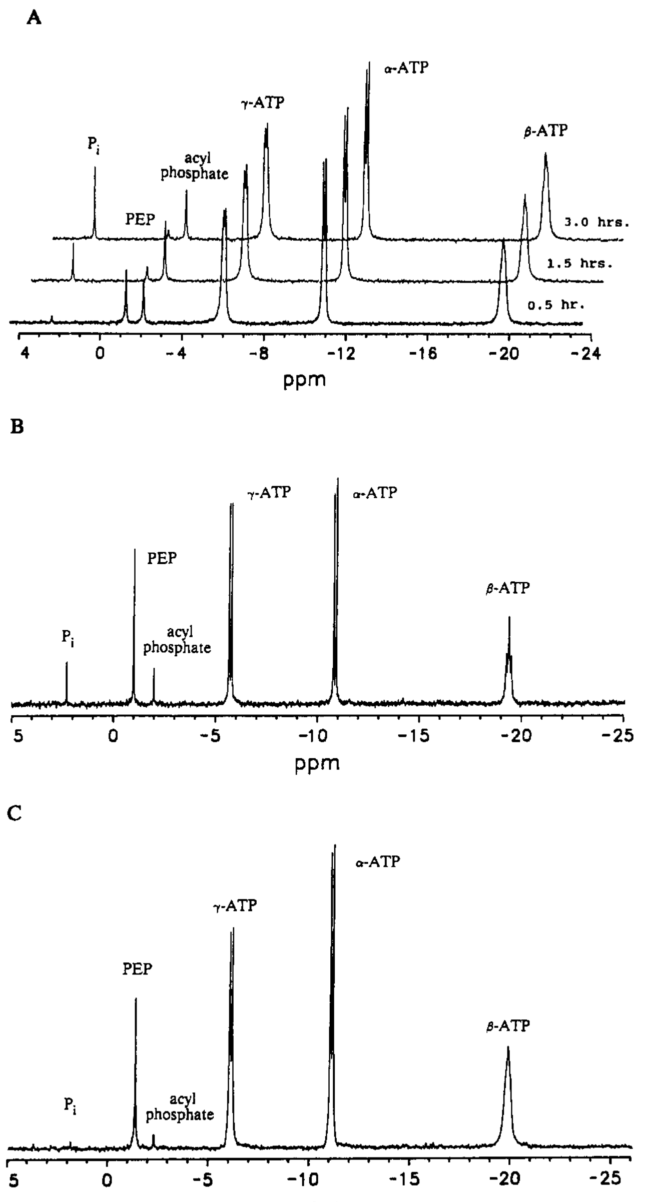


FIGURE 2: ^{31}P NMR spectra for the reaction of L-aspartate or its analogues with aspartokinase I at 6 °C. The reaction mixture contained 100 mM Hepes(Tris), pH 8.0, 100 mM KCl, 30 mM MgATP, 15–40 mM amino acid, and 3 units of AK-HSD I. The ATP was recycled with 30 units of pyruvate kinase and 15 mM phosphoenolpyruvate. (A) Time course with L-aspartic acid; (B) L-aspartate β-methyl ester after 12 h; (C) L-asparagine after 14 h.

Table III: Phosphorus-31 NMR Chemical Shifts of Phosphorylated Species

compound	δ^a (ppm)
β-aspartyl phosphate	−2.07
β-2-methyl-DL-aspartyl phosphate	−2.04
α-phospho-L-aspartate β-methyl ester	−2.25
β-phospho-L-aspartate α-benzyl ester	−2.13[b]
L-asparaginyl phosphate	−2.11
ATP, γ-phosphate	−5.71[c]
ATP, α-phosphate	−10.84[c]
ATP, β-phosphate	−19.36[d]
phosphoenolpyruvate	−0.96
P_i	2.41

[a] Chemical shifts were determined at pH 8 and are reported relative to 85% phosphoric acid as an external standard. [b] A peak arising from a contaminant in the benzyl ester was observed at δ −1.92. [c] Doublet, 15.5 Hz coupling constant. [d] Triplet, 15.5 Hz coupling constant.

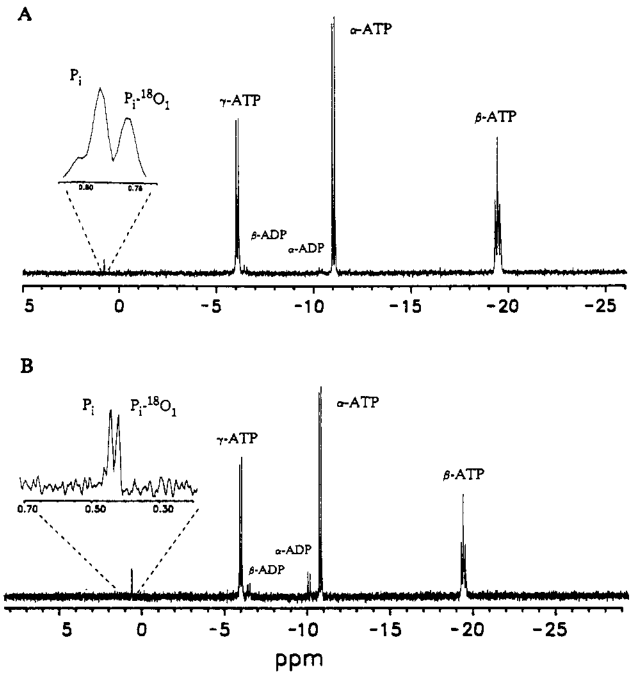


FIGURE 3: Incubation of L-aspartic acid after 16 h (A) or L-aspartate β-methyl ester after 60 h (B) with aspartokinase I in 50% oxygen-18-labeled water. Reaction conditions: 100 mM Hepes(Tris), pH 7.0, 100 mM KCl, 6 mM MgATP, 27 μg of AK-HSD I, and 15–40 mM amino acid.

and corroborate the absence of an esterase activity in AK-HSD I.

An isotope labeling approach was utilized to gain some insight into the mechanism of action of aspartokinase I. For these studies the reactivity of the analogues of L-aspartic acid was assessed on the basis of the accumulation of stable inorganic phosphate. Mechanistic information was obtained by monitoring the transfer of the oxygen-18 label from the substrate to inorganic phosphate, or from $H_2{}^{18}O$ to inorganic phosphate via an acyl phosphate intermediate. Oxygen-18 is known to induce an upfield shift in both ^{13}C and ^{31}P NMR signals (Cohn, 1982; Van Etten & Risley, 1981) and can therefore be a useful indicator of the site of bond breaking during the hydrolysis of the acyl phosphates. Oxygen-18-labeled L-aspartic acid prepared according to the procedure of Khazaeli and Viola (1984) showed an upfield shift of 0.025–0.026 ppm/oxygen-18 substitution at the carboxylic carbons. Three peaks were observed for each of the carboxylic carbons of the labeled aspartic acid corresponding to the following species: [α,β-$^{16}O_2$]L-aspartic acid (58%), [α,β-$^{16}O^{18}O$]L-aspartic acid (35%), and [α,β-$^{18}O_2$]L-aspartic acid (7%). When the aspartokinase reaction was run using this labeled compound at pH 8.0, an ^{18}O-labeled inorganic phosphate peak was observed 0.027 ppm upfield from the unlabeled species.

In a complementary series of experiments, conducted using unlabeled L-aspartic acid or its analogues, the transfer of label was monitored from $H_2{}^{18}O$ to inorganic phosphate through the hydrolysis of the unstable acyl phosphate products. At pH 7.0, incorporation of the ^{18}O isotopic label into P_i was observed for L-aspartic acid (Figure 3A). Similar oxygen-18 incorporation from water was observed with the β-derivatized substrate analogues, such as L-aspartate β-methyl ester (Figure 3B). In contrast, only unlabeled inorganic phosphate was observed to accumulate for L-aspartic acid and L-aspartate β-methyl ester at pH 8.0 and above. The spectrum for the reaction with L-cysteine sulfinate was unchanged from pH 7 to pH 9, with only unlabeled inorganic phosphate produced.

DISCUSSION

Binding Specificity of Aspartokinase I. The specificity of aspartokinase I has been examined to determine the structural features that are required for binding and catalysis. The enzyme is quite specific for the nucleotide substrate with only ATP, among the common nucleotides, functioning as a phosphoryl donor. 2′-Deoxy-ATP was found to be an alternative nucleotide substrate, with kinetic parameters comparable to ATP, and β,γ-methylene-ATP is a competitive inhibitor vs ATP (Angeles & Viola, 1990).

An intact α-amino group is absolutely essential for productive binding of the amino acid substrate as a phosphoryl acceptor. In fact, no interaction with the enzyme was detected when N-substituted aspartic acids were examined either as possible alternative substrates or inhibitors. However, the enzyme allows considerably wider latitude for the carboxyl-binding determinants of the amino acid substrate. The distance between the carboxyl groups does appear to be critical for productive binding. Structural analogues in which this distance is either longer (L-glutamate and 3-aminoglutarate) or shorter (2-aminomalonate) are not accepted as alternative substrates. Both of these compounds bind as competitive inhibitors, but with affinities that are about 25-fold weaker than the normal substrate. However, L-norvaline, a glutamate analogue in which the γ-carboxyl group is replaced by a methyl group, binds to the enzyme an order of magnitude more tightly than either of these compounds. The altered position of the R-carboxyl group in L-glutamate and 2-aminomalonate must interfere with the normal substrate binding interactions. This interference is apparently relieved by the neutral side chain of L-norvaline. This critical spacing between the active site groups that interact with the substrate carboxyl groups is also confirmed by the structural analogues of β-aspartyl phosphate that bind to the enzyme. Homoserine phosphate and 5-phosphono-2-aminovalerate, compounds that have the same number of atoms in the linear array as β-aspartyl phosphate, are more potent inhibitors than *O*-phospho-L-serine and 4-phosphono-2-aminobutyrate. The α-carboxyl group is also an important binding determinant for the amino acid substrate. Structural analogues with derivatized α-carboxyl groups, such as α-amides or α-esters, have a weaker affinity for the enzyme than underivatized L-aspartic acid. However, in contrast to compounds with derivatized α-amino groups, the analogues with derivatized α-carboxyl groups bind in the same orientation as the underivatized substrate, as evidenced by their ability to function as alternative substrates for aspartokinase I. The binding pocket in that region of the active site must be quite large, since even analogues containing a bulky substituent such as an α-benzyl ester can be accommodated as a substrate without a substantial loss in either binding affinity or catalytic efficiency.

Alternative Substrates. The finding that some α-carboxyl derivatives of L-aspartic acid can function as phosphoryl acceptors in the aspartokinase-catalyzed reaction is not particularly remarkable. The α-amino group has already been shown to be an essential binding group and can serve to orient these alternative substrates in a comparable manner to the normal substrate. However, the discovery that structural analogues with a derivatized β-carboxyl group could also serve as alternative substrates was unexpected, since this is the nucleophile in L-aspartic acid that attacks the γ-phosphoryl group of ATP to effect phosphoryl transfer. The range of β-carboxyl derivatives of aspartic acid that were found to function as alternative substrates for aspartokinase includes the β-amide, β-hydroxamate, β-esters and even an analogue in which the carboxyl group is replaced with a sulfinate group. Several hypotheses have been examined to arrive at an explanation for these unusual findings.

It was considered unlikely that the enzyme would possess an inherent ability to catalyze the hydrolysis of this wide range of derivatives to yield the underivatized aspartic acid as the product. This was confirmed when L-aspartate dimethyl ester was examined as a putative substrate. The diester does interact with the enzyme as a relatively weak competitive inhibitor. However, once a small level of contaminating monoester was removed, no evidence of catalytic turnover was observed with this diester or with the diethyl ester.

The possibility that these β-carboxyl derivatives were not actually being phosphorylated would provide a reasonable explanation of the results that have been observed. The initial determination of the substrate activity of these alternative substrates had been made with a coupled assay for ADP production. These aspartate analogues could bind to aspartokinase in a catalytically viable ternary complex with ATP. However, since the β-carboxyl group is not available as a phosphoryl acceptor, the γ-phosphoryl group of ATP could be attacked by an alternative nucleophile such as a solvent water molecule. During the normal catalytic cycle with L-aspartic acid as the substrate, it would certainly be advantageous for the enzyme to be able to exclude solvent from the active site. However, the structural changes in these analogues, compared to the normal substrate, may cause some alterations in the binding orientation at the active site that are sufficient to allow access to solvent. This sort of phenomenon is not without precedent. Aldehydic derivatives of sugar substrates have been found to induce a phosphoryl transfer to water (ATPase activity) in the reactions catalyzed by hexokinase, fructokinase, and phosphofructokinase (Viola & Cleland, 1980; Rendina & Cleland, 1984). To distinguish between the substrate carboxyl group and solvent water as the phosphoryl acceptor is not as straightforward as it first appears. The normal product of the phosphoryl transfer, β-aspartyl phosphate, is unstable in aqueous solution. The products of this nonenzymic hydrolysis are aspartic acid and phosphate, the same products that would be observed if the phosphoryl group was directly transferred to water. If the acyl phosphates produced from these alternative substrates were considerably less stable than β-aspartyl phosphate, it would be quite difficult to observe the initial product of the enzymic reaction.

Several different approaches were used to address this problem. First, the substrate activity of these substrate analogues was examined with a coupled assay for the acyl phosphate products. The enzyme aspartate-β-semialdehyde dehydrogenase has been found to have rather broad specificity for the acyl phosphate substrate (T. Angeles, unpublished results). Virtually all of the analogues that led to ADP production in the presence of aspartokinase were also found to produce acyl phosphates that were substrates for aspartate-β-semialdehyde dehydrogenase, the next enzyme in the biosynthetic pathway. The only exceptions are cysteine sulfinate and 2-methylaspartate, which were not substrates in this coupled assay.

To provide further evidence supporting the proposed transfer of a phosphoryl group to these β-derivatized substrate analogues, these reactions were examined using oxygen-18-labeled reactants. The labeling pattern observed upon phosphorylation of aspartic acid, and the other substrate analogues, followed by the subsequent hydrolysis of the products, changes in going from pH 7 to pH 9. This change reflects a pH-dependent change in the mechanism of hydrolysis of acyl phosphates. At neutral pH the mechanism of hydrolysis is the attack of water at phosphorus, with P–O bond cleavage resulting in the displacement of the carboxylate to yield free aspartic acid (Koshland, 1952). When this reaction is carried out in oxygen-18-labeled water, an atom of oxygen-18 is incorporated into the inorganic phosphate generated by this hydrolysis. At higher pH, hydroxide becomes the attacking species, and carbonyl compounds are known to be highly reactive toward strong nucleophilic reagents. This change in the site of attack to the carbonyl carbon results in C–O bond scission and subsequent displacement of phosphate (Koshland, 1952). Under these conditions, oxygen-18 is incorporated into the carbonyl group releasing unlabeled inorganic phosphate. However, if the labeled oxygen is introduced into the carboxyl group of the substrate, then hydrolysis by this base-catalyzed mechanism results in the observed transfer of this label to the departing phosphate group.

The stability of the acyl phosphates derived from these alternative substrates was examined by enzymic end-point assays. While most of these products were somewhat less stable than β-aspartyl phosphate, the half-lives were sufficiently long to allow the possibility of direct detection if the reactions were examined at lower temperatures with high levels of aspartokinase. The production of acyl phosphates was examined by monitoring these reactions by phosphorus-31 NMR spectroscopy. In each case, conditions were found to allow sufficient accumulation of the product for detection of a singlet in the region expected for phosphate monoesters (Cozzone & Jardetzky, 1976). Even 2-methylaspartate was found to lead to the production of an acyl phosphate. However, this product is apparently not a substrate for ASA-DH, confirming the observations made earlier by Shames et al. (1984). Examination of the phosphorylation of cysteine sulfinate in oxygen-18-labeled water did not lead to incorporation of oxygen-18 into either inorganic phosphate or ADP. This observation does not support the induction of an ATPase activity upon binding of cysteine sulfinate to aspartokinase. The failure to directly detect an acyl phosphate derived from cysteine sulfinate may simply reflect a low stability for this product.

The unavoidable conclusion, based on the observation of the products obtained from these alternative substrates, is that substrate analogues of L-aspartic acid that are derivatized at the β-carboxyl group are capable of productive binding to aspartokinase through a reversal of regiospecificity. The resulting α-acyl phosphates, produced by enzyme-catalyzed phosphoryl transfer, are, in most cases, substrates for aspartate-β-semialdehyde dehydrogenase, the next enzyme in the biosynthetic pathway. The products of that reaction, presumably the corresponding α-semialdehydes, also appear to be substrates for the next biosynthetic reaction catalyzed by homoserine dehydrogenase (T. S. Angeles and R. E. Viola, unpublished results). These observations raise the possibility of using these enzyme-generated alternative substrates with reversed regiospecificity to synthesize antimetabolites for the enzymes that are downstream in this amino acid biosynthetic pathway.

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