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Purification and Characterization of the Recombinant *Arabidopsis thaliana* Acetolactate Synthase

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Acetolactate synthase was purified from *Escherichia coli* MF2000/pTATX containing *Arabidopsis thaliana* acetolactate synthase gene. Purification steps included DEAE cellulose ion exchange column chromatography, phenyl sepharose hydrophobic column chromatography, hydroxylapatite affinity column chromatography, and Mono-Q HPLC. Molecular weight was estimated to be ~65 KDa and purification fold was 109 times. The enzyme showed a pH optimum of 7 and the K_M value was 5.9 mM. The purified enzyme was not inhibited by any of the end products, valine, leucine, and isoleucine.

Introduction

Acetolactate synthase (ALS) is the first common enzyme in the biosynthetic pathways leading to the branched chain amino acids leucine, isoleucine, and valine. It condenses an acetaldehyde moiety derived from pyruvate either with another molecule of pyruvate to form 2-acetolactate or with 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate. ALS has been conserved across species boundaries and substantial sequence homology are observed between the enzymes of bacteria, yeast, and higher plants.[1] Animals do not have the branched-chain amino acid pathways, and therefore, must ingest these amino acids in the diet. In *Escherichia coli* and *Salmonella typhimurium*, three ALS isozymes have been characterized.[2] In these enterobacteria, ALS occurs as a tetramer of two large and two small subunits. The genes encoding each isozyme from *E. coli* had been cloned and sequenced.[3-5] Genes coding for proteins homologous to the large subunit of bacterial ALS have been cloned and sequenced from yeast *Saccharomyces cerevisiae*[6,7] and from the higher plants *Arabidopsis thaliana* and *Nicotiana tabacum*.[1] No small subunit has been demonstrated to be necessary for catalytic activity of either the yeast or the plant ALS enzymes. In addition, regulation of the biosynthesis of valine, leucine and isoleucine in plants is still not fully understood.[8]

ALS is the target of six classes of structurally unrelated herbicides, sulfonylureas,[6,9] imidazolinones,[10] triazolopyrimidines,[11] N-phthalylvaline anilide,[12] sulfonylcarboxamide,[13] and pyrimidyl-oxy-benzoate.[14] ALS enzymes from a wide range of organisms are sensitive to these compounds. Mutants resistant to the herbicides have been described in *Lolium rigidum*,[15-17] *Chlorella emersonii*,[18-20] and *Xanthium strumarium*,[21-23] and resistance has been shown to be attributable to an altered ALS enzyme.

The small crucifer *Arabidopsis thaliana* is commonly

used as a model system to study gene expression in plants.[24] The single gene encoding ALS in *Arabidopsis thaliana* shows extensive homology to the cloned ALS gene from another higher plant, *Nicotiana tabacum*.[1] The genes are about 2 kilobases long each, lack introns, and are approximately 85% homologous when the deduced amino acid sequences of mature enzyme are compared. Although the anabolic ALS is generally a labile enzyme, it has been purified from overexpressing or recombinant strains of *E. coli, Salmonella typhimurium*, and *Saccharomyces cerevisiae*.[25-28] However, the enzyme from plant has not been purified satisfactorily from wild plants or recombinant strains. In 1988, Durner and Boger reported the purification of ALS from barley.[29] But in 1991 Singh *et al.* reported that the purified ALS from barley reported by Durner and Boger was just a main impurity.[30]

In this report, we described a purification method of ALS enzyme of *Arabidopsis thaliana* from recombinant strains of *E. coli*. In addition, general characteristics of the cloned enzyme was examined.

Experimental

Materials

DEAE-cellulose, phenyl sepharose, flavine adenine dinucleotide (FAD), thiamine pyrophosphate (TPP), ampicilline, 3-β-indole acrylic acid (IAA), sodium pyruvate were obtained from Sigma Chemical Co. Sodium chloride, ammonium sulfate, potassium phosphate were obtained from Merck. Hydroxylapatite was obtained from Bio-Rad. All other chemicals were reagent grade commercially available.

Bacteria and bacterial growth condition

E. coli strain MF2000 containing the *ilv*B800 plasmid pTATX was donated by Dr. B. J. Mazur of E. I. du Pont de Nemours & Co. This recombinant *E. coli* resulted in the expression clone pTATX, in which the plant ALS coding sequences are situated downstream of a hybrid bacterial Trp promoter/operator region and ribosome binding site.[31] Bacteria were grown in LB medium base.[32] Ampicilline (100 mg/mL) was added to the medium used for propagating bacteria containing pBR322-derived plasmids. The inducing agent, 3-β-indole acrylic acid, was added at optical density (600 nm) of 0.7. The cells were harvested by centrifugation after 4 hours following the addition of the inducer and stored at − 70 °C.

Acetolactate synthase assay

The ALS activity was measured by estimation of the product, acetolactate, after conversion by decarboxylation in the presence of acid to acetoin.[33] Standard reaction mixtures contained the enzyme in 50 mM potassium phosphate buffer (pH 7.0) containing 200 mM sodium pyruvate, 10 mM $MgCl_2$, 1 mM thiamin pyrophosphate (TPP), and 10 mM flavin adenine dinucleotide (FAD). The total volume of reaction mixture was 1 mL. This mixture was incubated at 37 °C for 20 min after which time the reaction was stopped with addition of H_2SO_4 to make a final concentration of 0.85% H_2SO_4 in a microcentrifuge tube. The reaction product was allowed to decarboxylate at 60 °C for 20 min. The acetoin formed was determined by incubation with creatine (0.17%) and 1-naphthol (1.7% in 4 N NaOH). Maximum color was observed by incubation at 60 °C for 15 min followed by further incubation at room temperature for 15 min. The absorption of color complex was measured at 525 nm. One unit is defined by 1 μmol acetolactate produced per minute in the assay.

Purification of acetolactate synthase

Step 1. DEAE-cellulose ion exchange chromatography. All operation were carried out at 4 °C. Frozen cells of 12 g were thawed and suspended uniformly in 37.5 mL of standard buffer (20 mM potassium phosphate buffer, pH 7.0, containing 1 μM FAD, 1 mM $MgCl_2$, 0.5 mM β-mercaptoethanol, 0.1 mM thiamine pyrophosphate, and 20% glycerol (v/v)). The cells were disrupted by sonication. Sonication power was applied in 30 sec bursts with intermittent intervals for cooling of 2 minute until the suspension cleared, usually after 10 times of 30 sec bursts. The crude extract was then subjected to centrifugation at 100,000 × g for 60 minutes and the supernatant fluid was directly loaded to 3.9 × 25 cm column of DEAE-cellulose equilibrated with the standard buffer without FAD. The column was eluted with 1.2 L of linear NaCl gradient from 0 to 1 M.

Step 2. Phenyl sepharose hydrophobic chromatography. Active fractions were pooled and mixed with high concentration ammonium sulfate solution to make 0.5 M solution. This solution was applied directly to 2 × 6 cm column of phenyl sepharose CL-4B previously equilibrated with the standard buffer containing 0.5 M ammonium sulfate. In this column, large amount of protein was bound except the ALS. The non-bound fractions were mixed with saturated ammonium sulfate solution to make 1 M ammonium sulfate solution finally. This solution was applied again to another 2 × 6 cm column of phenyl sepharose CL-4B previously equilibrated with standard buffer containing 1 M ammonium sulfate. The phenyl sepharose CL-4B column was developed with 100 mL linear ammonium sulfate gradient from 1 to 0 M, and then washed with 2 volumes of the standard buffer.

Step 3. Hydroxylapatite affinity chromatography. The active fractions were pooled and applied to 1.5 × 5 cm column of hydroxylapatite. The column was washed with 100 mL standard buffer and continuously with 100 mL standard buffer containing 1 M NaCl. Finally this column was developed with 100 mL linear phosphate gradient from 0.02 to 0.12 M.

Step 4. HPLC Mono-Q chromatography. The active fraction was pooled and dialyzed for 1 hour against 20 mM Tris buffer (pH 7.0). This sample was applied to a Mono-Q HPLC anion exchange column which was previously equilibrated with 20 mM Tris buffer (pH 7.0). After the column was washed with 20 mM Tris buffer (pH 7.0) and developed with linear NaCl gradient from 0 to 0.4 M. Volume of each fraction was 0.5 mL and the pressure of HPLC was 35 bar.

Other method

The protein content of ALS preparation was determined according to Bradford.[34] PAGE was conducted using 10% polyacrylamide gel and 0.1% SDS and proteins were stained with silver nitrate.[35]

Results and Discussion

Purification of acetolactate synthase

DEAE-cellulose ion exchange chromatography. Since several purification cases of ALS from other species were reported that ammonium sulfate fractionation was used at the first step, ammonium sulfate fractionation was tested.[25,26] However, *Arabidopsis thaliana* ALS was poorly recovered from ammonium sulfate fractionation. Owing to the low recovery of ammonium sulfate fractionation, the chromatography of DEAE cellulose ion exchange was employed at the first step. Figure 1 shows the elution profile of acetolactate synthase activity and protein concentration from the chromatography of DEAE cellulose ion exchange. The activity was eluted around 300 mM NaCl in 0-1 M NaCl linear gradient. This step enriched the enzyme about five folds and had an unexpected recovery of 120%.

Phenyl sepharose hydrophobic chromatography. Subsequent chromatography on phenyl sepharose CL-4B achieved high enrichments due to the hydrophobicity of the enzyme. In this step, two successive columns of phenyl sepharose were used with different conditions. In the first column of phenyl sepharose, the column was equilibrated with the standard buffer containing 0.5 M ammonium sulfate and the sample loaded was also diluted with the standard buffer containing 0.5 M ammonium sulfate. In this condition most of ALS did not bind to phenyl sepharose column. However, it was possible to eliminate a large amount of proteins because of nonspecific binding of proteins to the column. This step was essential to obtain the final purity of the enzyme. The second column of phenyl sepharose was equilibrated with 1 M ammonium sulfate and the sample loaded was also diluted with the standard buffer containing 1 M ammonium sulfate. ALS bound to phenyl sepharose column was eluted at washing fraction with the standard buffer without ammonium sulfate. Figure 2 shows the elution profile of phenyl sepharose chromatography. The ALS activity was detected at washing fraction with the standard buffer. The activity peak was eluted a little later than the major protein peak. In this step, the enzyme purity increased almost 7 times. This step also has the advantage of offering the sample with low salt.

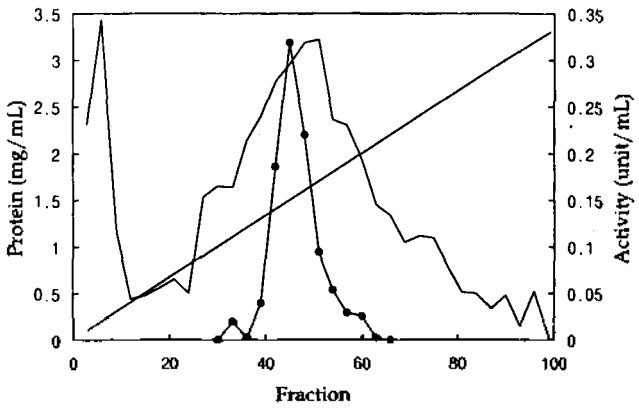

Figure 1. The elution profile of acetolactate synthase from DEAE cellulose column. The slope is 0-1 M NaCl gradient. The volume of each fraction was 8 mL. ——, protein; —●—, enzyme activity.

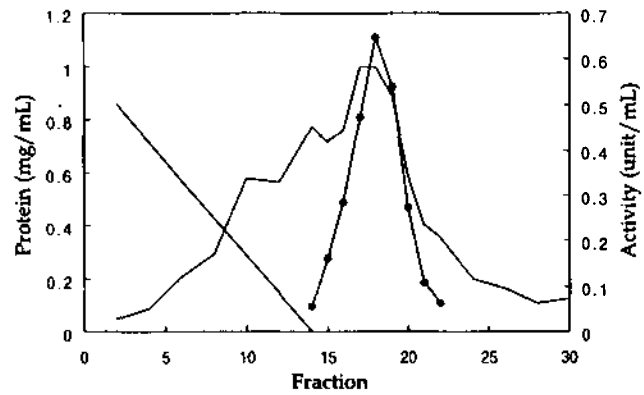

Figure 2. The elution profile of acetolactate synthase from the 2nd column of phenyl sepharose. The reverse slope was 1-0 M ammonium sulfate gradient. The volume of each fraction was 8 mL. ——, protein; —●—, enzyme activity.

Hydroxylapatite affinity chromatography. The third column used was the hydroxylapatite chromatography. Since the chromatographic separation was due to the properties of hydroxylapatite column, several methods were introduced. If protein has a phosphate dependent region and binds to the column, the elution could be managed as follows. Any protein with isoelectric point of 7.0-7.6 could be eluted by NaCl solution. If proteins were basic, then $MgCl_2$ solution could be used for elution. If the protein of interest was acidic protein, then a high concentration of phosphate solution can elute that protein.[36] In this experiment, as shown in Figure 3, two cases of elution methods, NaCl elution and phosphate elution, were performed. NaCl gradient 0-1 M or 1 M stepwise elution did not elute the ALS. In contrast, phosphate gradient elution revealed two peaks of ALS activity. In initial approach, $MgCl_2$ solution had been tested. But the assay of ALS showed abnormal behavior because Mg^{2+} act as a cofactor for ALS. The separation of two activity peaks of ALS in the Figure 3 was thought to be due to the rapid change of medium from sodium chloride to phosphate. When phosphate gradient was applied, ALS and other proteins were eluted conglomerately and

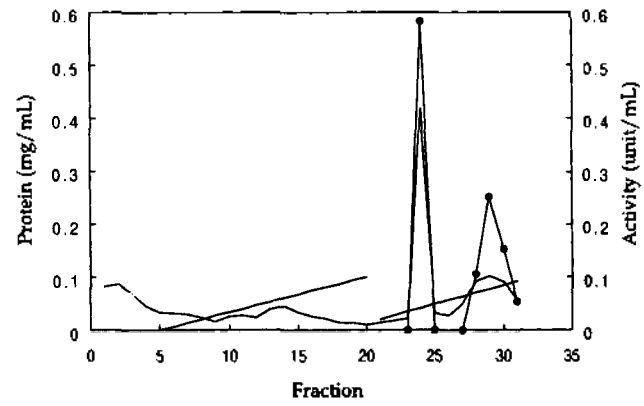

Figure 3. The elution profile of chromatography of hydroxylapatite affinity column. The 1st slope was NaCl gradient 0-1 M. The 2nd slope was phosphate pH 7.0 gradient of 0.02-0.12 M. The volume of each fraction was 8 mL. ——, protein; —●—, enzyme activity.

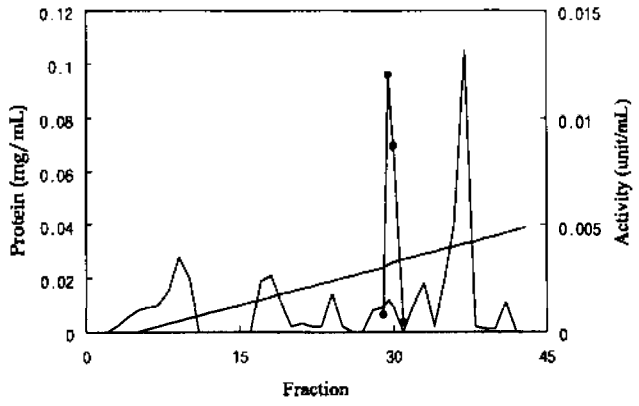

Figure 4. The elution profile of acetolactate synthase from HPLC Mono-Q column. The volume of each fraction was 0.5 mL. ——, protein; —●—, enzyme activity.

then ALS was eluted again at 0.06-0.07 M phosphate concentration. Usually, an observation of separation of activity peak in chromatography implies a possibility of isomers. However, isoforms of ALS could not be accounted for in this experiment, because *E. coli* MF2000 did not have any ALS gene and only pTATX plasmid had only one type of ALS. When we skipped the 1 M NaCl eluting step, ALS was eluted only at 0.06-0.07 M phosphate concentration as a single peak as expected. This step enriched the enzyme about three folds.

Mono-Q HPLC chromatography. The sample of fraction number 30 of Figure 3 was pooled and dialyzed against 20 mM Tris HCl (pH 7). The dialyzed sample was applied to Mono-Q HPLC column. Figure 4 shows the elution profile of ALS activity and protein concentration. The Mono-Q showed high resolved separation of ALS from other protein and gave a nearly purified ALS as shown in SDS-PAGE (Figure 5). However, this step of separation has critical shortcoming, because ALS activity was markedly decreased during dialysis. The final recovery of enzyme activity was very low and the specific activity barely improved. Nonetheless, the SDS-PAGE of ALS samples showed a nearly single protein band at a position corresponding to a molecular mass of 65 KDa (Figure 5). This position is

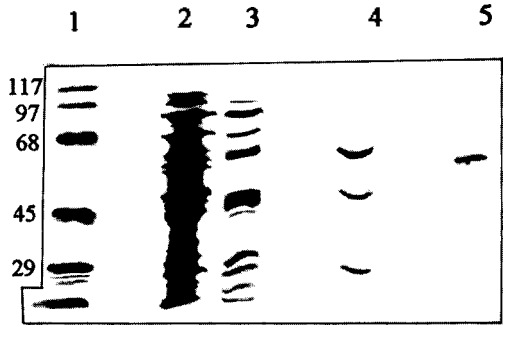

Figure 5. SDS/PAGE (10% acrylamide) of ALS at various purification stages. The gel was stained with silver nitrate to reveal the proteins. Lane 1, molecular marker 117, 97, 68, 45, 29 kDa; lane 2, phenyl sepharose active fraction; lane 3, hydroxylapatite active fraction; lane 4, 5 HPLC MONO-Q active fraction of 29 and 30 respectively.

Table 1. Summary of the purification procedure of ALS from 10 g of *E. coli* strain MF2000 containing pTATX plasmid

	Protein (mg)	Activity (U)	Yield (%)	Specific Activity (U/mg)	Purification Factor
Supernatant	3630	59.4	100	0.016	1
DEAE cellulose	876	71.3	120	0.0814	5
Phenyl sepharose	27	14.2	23.9	0.523	33
Hydroxylapatite	6	9.2	15.5	1.509	94
Mono-Q	0.012	0.02	0.04	1.75	109

One unit (U) was defined as the mmol of synthesized acetolactate per min (μmol/min).

similar to the report of Singh *et al.* by immunoassay.[30] A summary of the purification steps is shown in Table 1. A 109 fold purification was achieved from the high speed supernatant. The yield of overall procedure was about 0.04%.

General properties of purified ALS

General properties of the purified ALS such as pH dependence, substrate dependence, and time dependence were examined. ALS was assayed at several different pH values using four different buffer systems. As shown in Figure 6, a relatively sharp optimum pH at 7 was observed for the purified ALS. Result presented in Figure 7 shows that py-

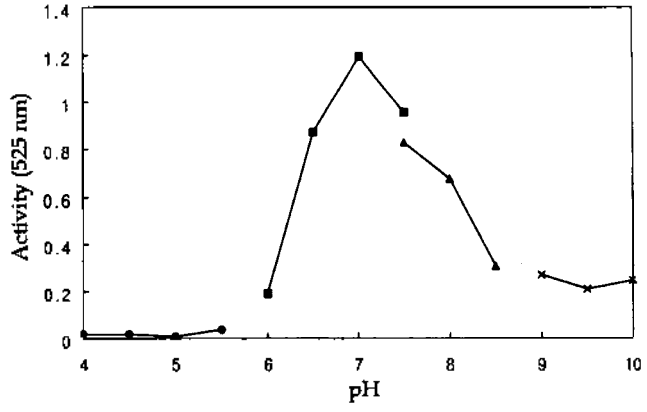

Figure 6. Effect of pH on acetolactate synthase activity. —●—, acetate; —■—, phosphate; —▲—, trycine; —×—, glycine

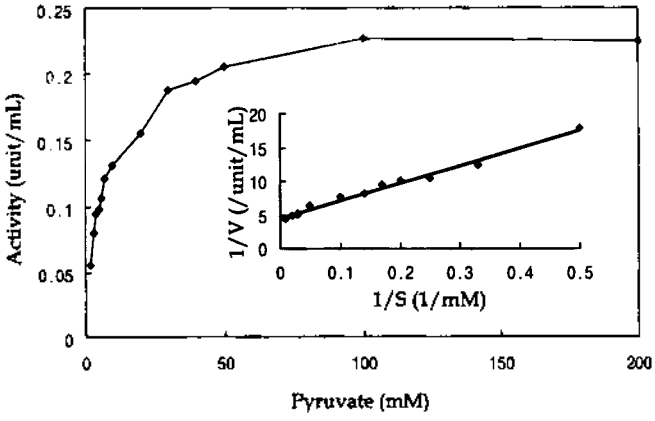

Figure 7. Dependence of acetolactate synthase activity on substrate pyruvate.

ruvate saturation curve is hyperbolic. The K_M for pyruvate was determined to be 5.9 mM. Similar results have been reported for enzymes from barley and black mexican sweet cells in the range of pyruvate concentration examined here.[37] At the standard assay condition, ALS activity was linearly proportional to the incubation time up to 60 min (data not shown). When effect of DMSO was examined since various inhibitors tested were usually dissolved in DMSO, ALS is activated up to 50% at 5% concentration of DMSO (data not shown). This observation is not surprising because DMSO in medium usually accelerates S_N2-type reactions and ALS is engaged to make two pyruvates to acetolactate by two S_N2-type reactions.[38]

Insensitivity of feedback inhibition

ALS from *Arabidopsis thaliana* seedling was sensitive to inhibition by the feedback inhibitors, valine and leucine. At the highest concentration of 1 mM for each amino acid, ALS activity from *Arabidopsis thaliana* seedlings was inhibited by about 60%.[39] In contrast, ALS activity extracted from *E. coli* MF2000 containing pTATX was insensitive to inhibition by all end products, valine, leucine and isoleucine (Figure 8). Assay of *Arabidopsis thaliana* enzyme in the presence of *E. coli* strain MF2000 did not change the property of the native plant enzyme.[39] However the *Arabidopsis thaliana* ALS expressed in *E. coli* did not have the sensitivity to valine and leucine in the *in vivo* experiment.[39] As expected the purified enzyme was inhibited markedly ($I_{50} \approx 2$ μM) by Cadre, an imidazolinone herbicide (data not shown). Therfore, the sensitivity toward herbicide seemed to be quite normal. A similar observation was noted in *Saccharomyces cerevisiae* where ALS was always partly desensitized to valine inhibition in the crude extracts, but 100% inhibition by valine was reached when the enzyme was assayed *in vivo* in benzene-permeabilized cells.[40]

Many possible reasoning for the insensitivity to feedback inhibitors of purified *Arabidopsis thaliana* ALS from *E. coli* could be suggested. It could be inability of the enzyme to adopt the right conformation at 37 °C. It could be due to an inability of the enzyme to aggregate to dimeric or tetrameric form. The other possibility is that second subunit that could be present in the plants is responsible for the feedback sensitivity of the enzyme. This possibility is supported by the observation made here that expression of the *Arabidopsis thaliana* ALS gene alone, which is similar to the large subunit of ALS in *E. coli*, yield an enzyme that is insensitive to inhibition by end products as shown in Figure 8. Therefore, the present results might be an indirect manifestation for a second subunit of ALS in plants. However, the subunit structure of ALS from higher plants has not yet been experimentally determined. The purification of the recombinant ALS from bacteria in a sufficient quantity will help to find out the structure and biochemical characteristics of the enzyme. Further studies on binding sites of herbicide will enable to design a new series of herbicides.

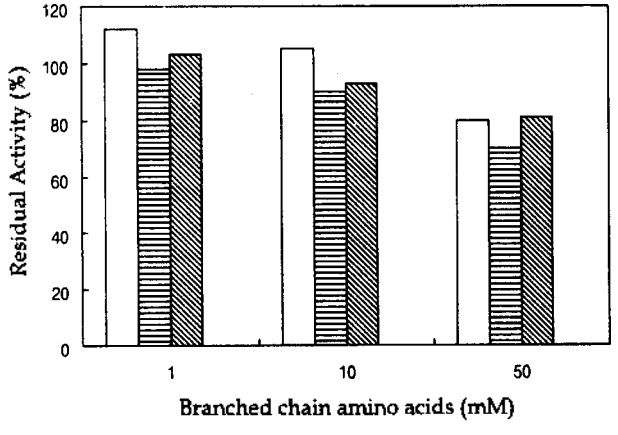

Figure 8. The effect of end products on the ALS activity. □, Val; ■, Leu; ▨, Ile.

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Protonation and Stability Constants for Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} of the Open-Chain Polyamine 1-Amino-13-(2-pyridyl)-3,6,9,12-tetraazatridecane. Crystal Structure of Its Nickel(II) Complex

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The new unsymmetric N_6 ligand 1-amino-13-(2-pyridyl)-3,6,9,12-tetraazatridecane (aptatd) containing one pyridyl group has been synthesized and characterized by EA, IR, and NMR. Its proton association constants (log K_H^n) and stability constants (log K_{ML}) for Co(II), Ni(II), Cu(II), and Zn(II) ions were determined at 298.1 K and ionic strength 0.100 mol dm^{-3} (KNO_3) in aqueous solution by potentiometry: log K_H^1=8.80, log K_H^2=8.49, log K_H^3=6.84, log K_H^4=4.17, log K_H^5=3.47; log $K_{ML}(Co^{2+})$=18.00, log $K_{ML}(Ni^{2+})$=21.31, log $K_{ML}(Cu^{2+})$=23.62, log $K_{ML}(Zn^{2+})$=15.60. The X-ray structure of its nickel(II) complex $[Ni(aptatd)](ClO_4)_2$ are reported: orthorhombic space group Pbca, a=15.715(1) Å, b=14.280(2) Å, c=19.443(2) Å, V=4363.4 (9) $Å^3$ with Z=8. The geometry around nickel is a distorted octahedron with the pyridine nitrogen atom being *cis* to the nitrogen atom of the terminal primary amine.

Introduction

Recent investigations in this laboratory have focused on the interaction of open-chain saturated polyamines containing pyridyl or imidazoyl groups with metal ions.[1,2] Martell *et al.* studied the interaction of pyridyl-containing polyamine ligands with a series of first-row transition metals and showed that the stability constants of the ligands are higher than those of the analogous aliphatic polyamines in spite of the weak σ-donating ability of the pyridyl group.[3-6] The saturated polyamines, which are soluble and stable in water, have been synthesized by hydrogenation of aldimino groups in Schiff bases.[1,2] It is to be expected that the hydrogenation will yield ligands which are much more flexible than the parent compounds and which thus can present their donor atoms to a metal from either a planar or a nonplanar arrangement.

In order to get further insight into the chemistry of the polyamines we have synthesized a new unsymmetric N_6 ligand 1-amino-13-(2-pyridyl)-3,6,9,12-tetraazatridecane (aptatd) as its pentahydrochloride salt. This potentially hexadentate ligand contains one pyridyl moiety and five aliphatic amines. Proton association constants and stability constants of the ligand with Co(II), Ni(II), Cu(II), and Zn(II) ions are determined by potentiometry and compared with those of analogous N_4 to N_6 ligands. And X-ray crystal