The Purification and Characterization of Arginase from
Saccharomyces cerevisiae*

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In Saccharomyces cerevisiae, ornithine transcarbamoylase and arginase form a regulatory multienzyme complex (Hensley, P. (1988) Curr. Top. Cell. Regul. 29, 35–75). In this complex, arginase acts as a negative allosteric effector for ornithine transcarbamoylase. Before an analysis of the factors which promote and stabilize complex formation, arginase was purified in milligram quantities from a plasmid-containing enzyme-overproducing, protease-deficient yeast strain and its physical characterization undertaken. The purified enzyme has a specific activity of 885 μmol urea min⁻¹ mg⁻¹ and a Kₘ for arginine of 15.7 mM. The ultraviolet spectrum has a maximum absorbance at 279 nm, and the steady-state fluorescence emission spectrum has a maximum intensity at 337 nm, suggesting that the 3 tryptophans/polypeptide chain are in a relatively hydrophobic environment. Arginase has a weakly bound manganese responsible for the maintenance of the catalytic activity and is known to be heat activated in the presence of manganese. This effect is half-maximal at 12.1 μM manganese. In addition to a catalytic requirement for manganese, the presence of a more tightly bound metal is suggested from sedimentation studies. The native trimeric enzyme has a sedimentation coefficient of 5.95 S. Removal of the weakly associated metal results in no change in the sedimentation coefficient. However, dialysis with EDTA causes the s-value to decrease to 4.65 S, suggesting that under these conditions, the trimeric enzyme may partially dissociate. An analysis of CD spectra shows that significant spectral changes result from the removal of both the weakly bound metal and dialysis against EDTA.

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The first committed step in the degradation of L-arginine in Saccharomyces cerevisiae is catalyzed by arginase (EC 3.5.3.1). Arginase catalyzes the hydrolysis of L-arginine to L-ornithine and urea. In yeast, arginase and ornithine transcarbamoylase (EC 2.1.3.3), which catalyzes the first committed step in the biosynthesis of arginine, are both present in the cytoplasm. Thus, in the presence of the active site ligands for ornithine transcarbamoylase and arginase, there is the potential for the coupling of the two pathways into a futile urea cycle resulting in the uncontrolled hydrolysis of ATP. To avoid this, arginase and ornithine transcarbamoylase form a multienzyme complex in which ornithine transcarbamoylase activity is inhibited while arginase remains active. Formation of the multienzyme complex results in the uncoupling of the two pathways and the elimination of the wasteful hydrolysis of ATP. This system was first characterized by Wiame and co-workers (Bechet and Wiame, 1965; Messenguy and Wiame, 1969; Wiame, 1971; Penninckx et al., 1974; Penninckx, 1975; Penninckx and Wiame, 1976) and has subsequently been studied in our laboratory (Eisenstein et al., 1984; Eisenstein, 1985; Duong et al., 1986; Eisenstein et al., 1986; Hensley, 1988).

An analysis of the three-dimensional structure of ornithine transcarbamoylase and arginase by electron microscopy demonstrated that both enzymes are trimers (Duong et al., 1986). These studies also showed that in the multienzyme complex, the two enzymes associate face-to-face with a one-to-one stoichiometry. Furthermore, equilibrium sedimentation studies demonstrated complex formation in solution, yielding a Kₛ of 23 nM, indicating that ornithine transcarbamoylase and arginase are tightly associated in the complex (Eisenstein et al., 1986). Although much is known about the structure and function of yeast ornithine transcarbamoylase and ligand-binding-promoted conformational changes in this enzyme which are required for complex formation (Eisenstein et al., 1984; Eisenstein, 1985; Eisenstein and Hensley, 1986; Eisenstein et al., 1986), less is known about the structural and enzymatic properties of yeast arginase.

The enzymatic activity of arginase from a number of sources has been shown to be manganese-dependent (Hirsch-Kolb and Greenberg, 1968; Hirsch-Kolb et al., 1970, 1971; Carvaljal et al., 1971; Munakata et al., 1976; Fujimoto et al., 1976; Stewart and Caron, 1977; Garganta and Bond, 1986). Preliminary evidence from our laboratory, using sedimentation equilibrium, suggested that release of weakly associated manganese simply by dialysis resulted in the reversible loss of enzymatic activity without a change in the quaternary structure of arginase. However, dialysis of arginase against chelating agents appeared to cause complete dissociation of the trimeric quaternary structure into monomers. It was of interest, therefore, to investigate the influence of metal ions on
the catalytic activity of arginase, as well as assess their impact on the tertiary and quaternary structure of the enzyme and determine their role, if any, in multienzyme complex formation.

To establish the role of metals in the structure and catalytic function of arginase, a rapid and reproducible protocol was developed that enabled the purification of milligram quantities of enzyme. Results presented in this study suggest that there may be two types of metals associated with yeast arginase. It is proposed that one type, presumably manganese, which is characterized by its relative ease of removal, is essential for catalysis and may play a small but significant role in stabilizing the quaternary structure of arginase. The second type of metal, whose identity has yet to be established, may be involved in stabilizing the tertiary and quaternary structure of arginase, since upon its release, large changes in circular dichroism and sedimentation coefficient relative to the native enzyme are observed.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Purification of Yeast Arginase**—Arginase was purified approximately 100-fold from the protease-deficient, arginase-overproducing strain of *S. cerevisiae* (22321). Fig. 1 shows the elution profile of arginase from the Green A-Sepharose column. Following a large breakthrough, arginase is eluted with a salt gradient at approximately 0.16 M NaCl. The specific activity is represented by the filled squares and runs with the first major peak following the breakthrough. The pooled fractions are denoted by the closed bar. Fig. 2 shows the elution profile of arginase from the Spherogel TSK-G3000 HPLC gel filtration column. Arginase elutes as a single, narrow peak. The second major peak following the breakthrough. The pooled fractions are again denoted by the closed bar. The summary of the purification protocol is presented in Table I. From 50 grams of cells, 10-20 mg of highly purified arginase are obtained. The specific activity ranges from 885 ± 60 units mg⁻¹. Homogeneity of arginase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fig. 3 shows Coomassie Brilliant Blue R 250 stained gels run as described under "Experimental Procedures". Arginase is represented by a single band.

**Spectroscopic Characterization**—The UV absorption spectrum of arginase shown in Fig. 4 is given as specific absorbance versus wavelength based on the extinction coefficient of ε̂279 nm = 0.71 cm² mg⁻¹ (Eisenstein, 1985). The spectrum is dominated by the absorbance of the 3 tryptophans/polypeptide chain (Sumrada and Cooper, 1984). Consequently, there is little fine structure observed from the 6 phenylalanines or 11 tyrosines in the amino acid sequence (cf. Wettlaufer, 1962). The fluorescence excitation and emission spectra for arginase are shown in Fig. 5. For the excitation spectrum, the emission was monitored at 337 nm. For the emission spectrum, the excitation was monitored at 295 nm. The emission spectrum has a wavelength maximum of 337 nm. The excitation spectrum has a wavelength maximum of 284 nm.

**Steady-state Kinetics Studies**—Fig. 6 shows the data from steady-state kinetics experiments represented as specific activity versus arginine concentration. The data were analyzed in terms of the Michaelis-Menten equation yielding a KM for arginine of 15.7 ± 1.3 mM and a Vmax of 887.5 ± 30.4 units mg⁻¹. The inset to Fig. 6 shows the specific activity plotted as a function of the log of the arginine concentration. This demonstrates that the Michaelis kinetics are observed over all arginine concentrations.

**Effect of Manganese on the Activity of Arginase**—To determine the role that manganese plays on the activation of arginase, manganese concentration was varied during the heat activation step ("Experimental Procedures"). Fig. 7 is a plot of the specific activity of arginase as a function of the manganese concentration used in the heat activation step. The data are described by a rectangular hyperbola. The activation is half-maximal at a manganese concentration of 12.1 μM, and the enzyme achieves maximum specific activity (845 units mg⁻¹) above 0.6 mM. The inset to Fig. 7 shows the specific activity plotted as a function of the log of the manganese concentration, demonstrating that the hyperbolic function maintains over a wide concentration range.

**Effect of Chelating Agents on the Structure of Arginase**—Velocity sedimentation experiments were undertaken to determine the role of metals on the structure of arginase. The sedimentation studies were done at different rotor speeds, 55,000 rpm for the two samples dialyzed against buffers without chelating agents and 60,000 rpm for arginase incubated with EDTA. The sedimentation coefficients were calculated using nonlinear least-squares analysis with multiple

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**TABLE I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
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<th>Amount*</th>
<th>Specific Activity*</th>
<th>Yield</th>
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<td>units</td>
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<td>423</td>
<td>16,870</td>
<td>10.025</td>
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<tr>
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<td>14.7</td>
<td>9,629</td>
<td>6654</td>
<td>100</td>
</tr>
</tbody>
</table>

*Protein concentration was determined by the method of Kalkkar (1947).

**Protein concentration was determined by the method of Kalkkar (1947).**
absorbance versus wavelength was recorded in 20 mM Hepes, pH 7.0, 200 mM NaCl, 2 mM β-mercaptoethanol, and 1 mM MnCl₂.

For the emission spectrum, the excitation was monitored at 295 nm. For the excitation spectrum, the emission was monitored at 337 nm.

independent variables. The values calculated for the sedimentation coefficients with 5.95 ± 0.11 S for the native trimer, 5.92 ± 0.04 S for the trimer without the catalytic manganese, and 4.65 ± 0.10 S for the enzyme treated with EDTA (data not shown).

In order to determine whether incubation with EDTA has any effect on the secondary structure of arginase, CD spectra were taken. Fig. 8 shows the CD spectra for arginase under the three different conditions described above. The lower curve is the spectrum of native arginase (filled squares). Removal of the weakly associated metal by dialysis causes a significant decrease in ellipticity (circles). Treatment of the enzyme by dialysis against EDTA promotes a further decrease in ellipticity (triangles). These curves were analyzed for secondary structure by the method of Provencher and Glockner (1981). Arginase, in the presence of exogenous manganese, has 15% α-helix, 39% β-sheet, 15% β-turn, and 32% residual structure. Arginase, in the absence of exogenous manganese has 15, 39, 17, and 29%, respectively. Dialysis against EDTA results in changes to 15, 46, 27, and 12%, respectively. Note that the spectrum for the fully unfolded enzyme in 6 M guanidinium chloride (open squares) is strikingly different than that observed for arginase in the presence of chelating agents (Fig. 8).

**DISCUSSION**

Previously, we have reported the purification and characterization of ornithine transcarbamoylase (Eisenstein et al., 1984) as well as an architectural study of ornithine transcarbamoylase, arginase, and complex formation (Eisenstein, 1985; Eisenstein and Hensley, 1986; Eisenstein et al., 1986; Duong et al., 1986; Hensley, 1988). For further studies, however, it was important to first investigate the biochemical attributes of arginase and develop a purification protocol that would routinely yield homogeneous material.

The summary of the newly developed purification procedure for arginase from S. cerevisiae strain 02232i is given in Table I. The procedure involves two chromatography steps which include ion-exchange and gel filtration columns. The ion-exchange column, Green A-Sepharose, which has been modified from the procedure used by Garganta and Bond (1986) for the purification of rat liver arginase, results in a large increase in specific activity of the enzyme. After a concentration step, an HPLC-gel filtration step is used which enables both high resolution and rapid separation of arginase from the remaining contaminants. Our protocol also takes advantage of the heat stability of arginase at 55 °C as well as the solubility of the enzyme under slightly acidic conditions. Using this protocol, up to 40 mg of highly purified arginase can be obtained from 100 g of plasmid-containing, arginase-overproducing, protease-deficient yeast cells. The high purity of the enzyme isolated from this protocol is demonstrated by polyacrylamide gel electrophoresis under denaturing conditions as shown in Fig. 3. Arginase yields a single band when stained with Coomassie Blue R-250. The band represents 5 µg of purified arginase and is located at approximately 37,000 daltons.

The protease-deficient background is instrumental in yielding an undegraded, purified protein. It was noticed that if manganese was omitted at all during the purification or if EDTA was added at any point in the purification, arginase activity was lost and could not be regained. Thus, two modes of protease inhibition are unavailable: the use of EDTA, a strong inhibitor of metal-requiring lysosomal enzymes and the use of higher pH buffers, because MnCl₂ is oxidized at a higher pH. Therefore, although the strain is protease-deficient, all steps must be carried out as quickly as possible and at 4 °C to ensure an undegraded, purified enzyme.

**Yeast arginase has been purified previously by Penninckx et al. (1974) and Eisenstein (1985). However, these protocols would not routinely yield large amounts of homogeneous enzyme required for the studies to determine the roles metal ions play in the maintenance of the catalytic function and the stabilization of the quaternary structure of arginase. Eisenstein (1985) previously established a purification protocol from a protease-deficient, arginase-overproducing strain of S. cerevisiae, 1C1931a. This protocol entailed three chromatography steps: an ion-exchange column, an affinity column, and a low pressure gel filtration step. Using this procedure, 2-10 mg of arginase could be purified from 140 g of 1C1931a cells in 1 week. Because strain 02232i expresses larger quantities of active enzyme than strain 1C1931a, we hoped to use previously developed methodology for the purification. Unfortunately, chromatography steps employed previously did not routinely yield homogeneous protein when using 02232i.

The maximal specific activity of arginase purified by this
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FIG. 6. Initial velocity versus arginine concentration. Initial velocity as a function of arginine concentration was measured in 0.1 M glycine, pH 9.5 and 1 mM MnCl$_2$ at 30 °C. Arginase concentration was 300 ng ml$^{-1}$. Inset shows the initial velocity versus the log of the arginine concentration.

FIG. 7. Urea formation versus manganese concentration. Urea formation as a function of manganese concentration in the heat activation of arginase was measured in 0.1 M glycine, pH 9.5 at 30 °C in the presence of 25 mM arginine. Inset shows urea formation versus the log of the manganese concentration. Arginase concentration was 300 ng ml$^{-1}$.

The effect of arginine concentration on the activity of arginase is shown in Fig. 6. The steady-state kinetics for arginine are hyperbolic and yield an apparent $K_m$ for arginine of 15.7 ± 1.3 mM. The apparent Michaelis constants reported for mammalian liver arginases are in the range of 6–20 mM (Hirsch-Kolb et al., 1970) thus, similar to yeast arginase.

Since there are 3 tryptophans/polypeptide chain in yeast arginase (Sumrada and Cooper, 1984), spectroscopy could be utilized to monitor both ligand- and metal-binding-promoted structural changes in arginase. The ultraviolet absorption spectrum of arginase, as shown in Fig. 4, is dominated by the absorbance of the 3 tryptophans in the polypeptide chain.
The spectrum is characterized by a maximum in absorbance at 279 nm and a shoulder at 290 nm, both typical of tryptophan absorbance. The fluorescence spectra of arginase, shown in Fig. 6, is characterized by an emission maximum of 337 nm, suggesting that the tryptophans in arginase are in a moderately hydrophobic environment. Preliminary results from fluorescence studies suggest that as the metals are released, the environments of the 3 tryptophans become more polar, and there is a shift to longer lifetimes, consistent with a more polar environment.

To determine the effect of manganese on the activation of yeast arginase, manganese concentration was varied during the heat activation step of the assay. The half-maximal concentration for the heat activation of arginase by manganese is 12.1 μM with a maximal specific arginase activity of 844.8 ± 32.5 units mg⁻¹. The V₅₀ for yeast arginase in terms of both manganese and arginine are similar. Hirsch-Kolb et al. (1970) report that the optimal manganese concentration for mammalian liver arginase activity is 40 mM. We find that such high concentrations of manganese actually inhibit yeast arginase activity by up to 80% depending on the concentration of arginase present during the heat activation step (data not shown). Furthermore, the concentration of manganese normally present in our heat activation (10 mM) also has an inhibitory effect on arginase activity.

In order to determine the role metal ions play in the stabilization of the tertiary and quaternary structure of yeast arginase, velocity sedimentation experiments were undertaken. Preliminary studies of Eisenstein (1985), using sedimentation equilibrium, suggested that arginase, incubated with EDTA, dissociated fully into monomers similar to the results of Carvajal et al. (1971, 1977). Velocity sedimentation studies of yeast arginase in the presence of exogenous manganese obtained a sedimentation coefficient of 5.95 S, whereas a sedimentation coefficient of 5.92 S is obtained when exogenous metals are removed by dialysis. This excellent agreement indicates no gross structural change ensues upon removal of the weakly associated metal ion. When the enzyme was incubated with EDTA, however, the sedimentation coefficient decreases to 4.65 S. A decrease in the sedimentation coefficient for arginase in the presence of EDTA may result from an increase in effective molecular volume of the enzyme, reflecting an increased frictional coefficient. Alternatively, this decrease in s-value could be attributed to a dissociation of the trimeric quaternary structure into constituent monomers. For proteins with similar shape and size to the folded arginase monomer, a sedimentation coefficient of approximately 3.5 S would be expected (Creighton, 1983). This analysis assumes that dissociated monomers maintain their folded conformation. It is more likely that if the monomers are present, they are partially or significantly unfolded. An unfolded polypeptide chain of 37,000 Da would sediment at a rate less than 3.5 S. Since the observed sedimentation coefficient of 4.65 S in the presence of EDTA is intermediate between that derived for the trimer and that expected for either a folded or unfolded monomer, the chelating agent may remove a structural metal that results in the destabilization of the trimer and promotes the dissociation of the trimers into monomers. Preliminary equilibrium sedimentation experiments indicate that in the presence of EDTA, arginase may exist in a monomer-trimer equilibrium.

Circular dichroism experiments were undertaken to determine the effect of removal of the weakly associated metal as well as the effect of chelating agents on the secondary structure of arginase. Fig. 8 demonstrates that there is an overall decrease in ellipticity as metal is removed. Analysis of the data by the method of Provencher and Glockner (1981) reveals, however, that arginase treated with exogenous manganese and arginase dialyzed against a buffer containing no metals retain similar amounts of each type of secondary structure. Incubation with EDTA results in an increase in both β-sheet and β-turn structures. The CD spectrum of the sample treated with EDTA, though, does not resemble that for the enzyme that has been treated with guanidinium chloride (Fig. 8).

Summary—A large scale purification protocol for arginase from S. cerevisiae has been developed and the physical characterization begun. This protocol yields a preparation of arginase which is highly purified (> 99%) and has a higher specific activity than that obtained previously in our laboratory (Eisenstein, 1985). Previous results suggested that treatment of arginase with EDTA caused the trimeric enzyme to dissociate fully into monomers. However, results presented here on an enzyme purified by a new purification protocol yielding a significantly higher specific activity show that treatment with EDTA results in only partial dissociation of arginase into monomers. In the absence of the weakly and strongly bound metals, the data suggest that arginase may exist in a monomer-trimer equilibrium. Thus, metals play an important role in maintaining both the catalytic activity of the enzyme as well as contributing to the stability of the tertiary and quaternary structure. Only the trimeric form of the enzyme has been shown to form the tight complex with ornithine transcarbamoylase (Eisenstein, 1985; Eisenstein et al., 1986; Duong et al., 1986). It remains to be determined what roles metals ultimately play in promoting multienzyme complex formation.

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a S. M. Green, A. Ginsburg, M. S. Lewis, and P. Hensley, manuscript in preparation.

b S. M. Green, A. Ginsburg, M. S. Lewis, and P. Hensley, manuscript in preparation.
REFERENCES

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SUPPLEMENTARY MATERIAL TO

PURIFICATION AND CHARACTERIZATION OF YEAST ARGINASE

Neuroendocrine Studies: Velocity and direction were measured with a Beckman Model G centrifugal ultracentrifuge equipped with a temperature and temperature control system. Electrophoresis was carried out with crosslinked type acrylamide and starch window. The data were analyzed using the program "gel."
Purification and Characterization of Yeast Arginase

The crude extract was adjusted to pH 7.0 by the addition of 1 M Tris buffer and placed in a beaker with a stirrer and a cooling bath. The solution was cooled to 4°C and then dialyzed against 50 mM Tris buffer containing 0.5 M NaCl at 4°C for 10 min. The solution was then stirred for an additional 5 min. The supernatant was collected and the sediment was discarded.

The supernatant was dialyzed against 50 mM Tris buffer containing 0.5 M NaCl at 4°C for 24 h. The solution was clarified by centrifugation at 10,000 x g for 30 min.

The supernatant was then applied to a column of Cibacron Blue 3G-A Sepharose. The column was eluted with a linear gradient of NaCl from 0 to 0.5 M in 50 mM Tris buffer at a flow rate of 2 ml/min. Fractions containing enzyme activity were pooled and dialyzed against 50 mM Tris buffer at pH 7.5.

The elution profile of the enzyme was monitored by measuring the absorbance at 280 nm. The enzyme activity was determined by the method of Goodwin and Johnson.

Figure 1. Elution of yeast arginase from Cibacron Blue A-Sepharose chromatography. Fractions of 1 ml were collected and assayed for arginase activity. Fractions 23-42 (dialyzed by half) were pooled and dialyzed against 50 mM Tris buffer at pH 7.5, and then concentrated to 1 ml with a SpeedVac concentrator. The resulting sample was stored at -80°C until used.

Figure 2. Elution of arginase from Sphero Gel-3000, pH 7.5. Fractions of 1 ml were collected and SDS polyacrylamide gel run to assess purity. Fractions containing and band (dotted by line) were collected, concentrated, and dialyzed against 50 mM Tris, pH 7.5, 200 mM NaCl, 1% SDS, and 1 M NaCl.

Figure 3. SDS polyacrylamide gel electrophoresis of purified arginase. Lane 1 contains 5 µg of yeast arginase, lane 2 contains the molecular weight standard from Bio-Rad, polyvinylpyrrolidone 6, 62,500 Da, bovine serum albumin, 66,200 Da, ovalbumin, 45,000 Da, carbonic anhydrase, 21,500 Da, and trypsin 14,400 Da. Samples in 1% SDS and 1% bromophenol blue were heated at 100°C for 5 min prior to electrophoresis.
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