Regulation of Arginine Metabolism in *Saccharomyces cerevisiae*

ASSOCIATION OF ARGINASE AND ORNITHINE TRANSCARBAMOYLASE*

(Received for publication, January 3, 1986)

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Association of arginase and ornithine transcarbamoylase (OTCase) has been proposed to play an essential role in the regulation of arginine metabolism in *Saccharomyces cerevisiae* (Wiaime, J.-M. (1971) Curr. Top. Cell. Reg. 4, 1–39). In this report multienzyme complex formation is directly demonstrated in the presence of the active-site ligands for OTCase and arginase. Using equilibrium sedimentation, a dissociation constant for complex formation was determined to be 2.3 × 10⁻⁸ M in the presence of ornithine and agmatine, active-site ligands for OTCase and arginase, respectively. A molecular stoichiometry in the complex of one molecule of OTCase to one molecule of arginase was verified using transmission electron microscopy. The dimensions of the complex were determined by negative staining and rotary and unidirectional shadowing techniques to be 102 Å wide by 81 Å high. These dimensions are quantitatively consistent with dimensions of the individual enzymes (Duong, L. T., Eisenstein, E., Green, S. M., Ornberg, R. L., and Hensley, P. (1986) J. Biol. Chem. 261, 12807–12813). The enzymatic activity of OTCase is virtually completely inhibited when associated with arginase, reflecting the dramatic modulation of enzyme activity as a consequence of the acquisition of quaternary structure in this multienzyme complex.

The molecular mechanisms for the operation and control of almost all cellular processes ultimately involve proteins and their specific interactions. Both intrinsic (those within or between subunits) and extrinsic (protein small-molecule) interactions are important in endowing protein ensembles with the ability to modulate these processes (Klotz et al., 1975; Creighton, 1983). These interactions are linked and are fundamental to the manifestation of regulatory function.

The process of regulating arginine metabolism in *Saccha-

romyces cerevisiae* is in part modulated in this manner. Quaternary interactions in a multienzyme complex, situated at the interface between the arginine anabolic and catabolic pathways, regulate the flux of metabolites through the biosynthetic pathway in response to changes in the levels of intermediates in arginine metabolism. This regulatory complex is composed of two enzymes: ornithine transcarbamoylase (OTCase¹), which catalyzes the first committed step in arginine biosynthesis, and arginase, which catalyzes the first committed step in its degradation. The association of these enzymes to form the regulatory complex is mediated by the binding of small-molecule, active-site ligands ornithine and arginine. In the absence of both ligands, these cytoplasmic enzymes are dissociated and fully active. Alternatively, in the presence of active-site ligands, the complex forms and the enzyme activity of OTCase is virtually completely inhibited, whereas the activity of arginase remains essentially unaffected (Messenguy and Wiaime, 1969; Messenguy et al., 1971; Penninkx, 1975; Penninkx and Wiaime, 1976). Hence, this system provides a dramatic example of the regulation of biological activity via the linkage between intrinsic and extrinsic protein interactions.

The aim of these experiments was to probe the architecture and energetics of the protein interactions within the macro-molecular assembly. Using homogeneous enzymes, equilibrium sedimentation, steady-state kinetics, and electron microscopy have been used to initiate a structural, kinetic, and thermodynamic investigation of the events leading to the modulation of OTCase enzyme activity in the complex.

EXPERIMENTAL PROCEDURES

Materials—All materials, including chemical reagents and enzymes, were prepared as previously described (Duong et al., 1986). Arginase and OTCase were purified from plasmid-containing, enzyme-overproducing yeast strains as described elsewhere (Eisenstein et al., 1984; Eisenstein, 1985).

Enzyme Assays—OTCase was assayed as previously described (Eisenstein et al., 1984). Arginase was assayed using a modification of the method of Schimke (1962, 1964; Eisenstein, 1985). The arginase binding-promoted inhibition of OTCase was demonstrated using purified enzymes in enzyme kinetics assays. Reactions included buffered solutions of OTCase and substrates, 50 mM ornithine and 2 mM carbamoyl phosphate, and varying concentrations of arginase (see legend to Fig. 7).

Electron Microscopy—Electron microscopy was performed on the multienzyme complex essentially as described for the individual enzymes (Duong et al., 1985, 1986). In this case, however, the enzymes were equilibrated in the presence of 60 mM ornithine and 10 mM

¹The abbreviations used are: OTCase, ornithine transcarbamoylase; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.
agmatine, a competitive inhibitor of arginase, prior to fixation and staining.

Equilibrium Sedimentation—Equilibrium sedimentation experiments were performed with a Beckman Model E analytical ultracentrifuge equipped with photoelectric scanner and temperature control system. Double sector cells with charcoal-filled epon centerpieces and quartz windows were used. Data were collected automatically with a PDP 11/03-L computer after attaining equilibrium, usually 36 h after reaching the desired speed.2

Sedimentation experiments were performed at 21.5 °C in 20 mM HEPES, pH 7.0, 50 mM ornithine, 1 mM MnCl₂, and 10 mM agmatine. To determine the apparent dissociation constant for multienzyme complex formation as discussed below, it was necessary to know the molecular weights and partial specific volumes for OTCase, arginase, and the multienzyme complex. For OTCase, the molecular weight and partial specific volume were 110,000 and 0.732 ml/g, respectively (Eisenstein et al., 1984). The molecular weight of arginase was calculated from equilibrium sedimentation to be 110,000 (Eisenstein, 1985) based on a partial specific volume of 0.731 ml/g (determined from Sumrosa and Cooper, 1985). The specific extinction coefficients for OTCase and arginase were 0.36 cm² mg⁻¹ and 0.71 cm² mg⁻¹, respectively (Eisenstein et al., 1984; Eisenstein, 1985). The apparent molecular weight was the sum of the molecular weights of the two enzymes. Partial specific volume and specific extinction coefficient for the multienzyme complex were assumed to be the weight average of the values for OTCase and arginase.

At sedimentation equilibrium, the concentration distribution of a single homogeneous species is given by \( \sigma = \sigma_0 \exp[\lambda(M(1 - \rho)\omega^2(\rho - \rho_0)/2RT)] \) (1)

In these equations, \( \sigma \) is the concentration of the molecule at a radial position \( r \) (cm) from the center of rotation, \( \sigma_0 \) is the concentration of the molecule at the meniscus, \( M \) is the molecular weight, \( \lambda \) is the partial specific volume, \( \rho \) is the solvent density, \( \omega \) is the angular velocity, \( R \) is the universal gas constant, and \( T \) is the absolute temperature.

In the analysis of this interacting system, in which one molecule of arginase associates with one molecule of OTCase to yield one molecule of multienzyme complex, the concentration distribution of all macromolecular species may be described by the sum of three exponentials given by

\[ \sigma_i = \sigma_{ci} \exp[\lambda(M(1 - \rho)\omega^2(\rho - \rho_{ci})/2RT)] \]

(2)

where \( \sigma_{0i}, \sigma_{A}, \) and \( \sigma_{C} \) correspond to the exponential terms as described in Equation 1. Since chemical equilibrium is maintained throughout the centrifuge cell, the concentration terms in equation (3) may be related by

\[ \sigma_{ni} = \sigma_{ni,0} + \sigma_{ni,1} + (\xi_{ni,0} \xi_{ni,1})/K \]

(3)

where \( K \) is the dissociation constant for complex formation. By combining Equation 2 and Equation 3, the primary data from sedimentation studies may be analyzed directly by the following equation:

\[ \sigma_i = \sigma_{ci,0} \exp[\lambda(M(1 - \rho)\omega^2(\rho - \rho_{ci,0})/2RT)] \]

(4)

where \( \sigma_{ci,0}, \sigma_{ci,1}, \) and \( K \) are the parameters to be evaluated.

This method of data analysis is complicated by the high correlation of the parameters sought, and may therefore yield an unreliable estimate of the equilibrium constant. However, since the molecular weights and partial specific volumes for OTCase and arginase are almost identical, and since the initial concentrations of the two enzymes in these experiments are equal, the concentrations of the free enzymes will remain equivalent at any radial position at equilibrium. This condition reduces the analysis from that for a mixed association to that for a monomer-dimer equilibrium. Under these conditions, the parameters of interest are less highly correlated, and the parameters were determined from the least squares analysis (Hensley et al., 1975a, 1975b; Saxena et al., 1985).

RESULTS AND DISCUSSION

It has been proposed that OTCase and arginase from S. cerevisiae associate to form a regulatory multienzyme complex in which OTCase enzyme activity is virtually completely inhibited, uncoupling the catabolic and anabolic pathways in this organism (Messenguy and Wiame, 1969; Wiame, 1971; Messenguy et al., 1971; Penninckx, 1975; Penninckx and Wiame, 1976). Although Penninckx and Wiame (1976) have utilized enzyme kinetics and gel filtration techniques to estimate the strength of the interaction of these two enzymes in the complex, direct proof for existence of the multienzyme complex was sought. The studies reported here were undertaken to characterize the protein interactions which result in the reversible inactivation of OTCase in the complex using homogeneous enzymes. Specifically, we have sought to establish the energetic and architectural attributes of this protein assembly using equilibrium sedimentation and electron microscopy.

Equilibrium Sedimentation—The method of choice for investigating the association of these two enzymes is equilibrium

![Figure 1](image1.png)

**FIG. 1.** Demonstration of the OTCase-arginase multienzyme complex using equilibrium sedimentation. The primary data from equilibrium sedimentation experiments as discussed under "Experimental Procedures" in the form of total absorbance versus radius is shown in the upper panel. Purified OTCase and arginase were mixed in a one-to-one ratio (300 µg to 300 µg) and were dialyzed extensively against 20 mM HEPES, pH 7.0, 50 mM ornithine, 1 mM MnCl₂, and 10 mM agmatine. The rotor speed was 12,008 rpm and the temperature was 21.5 °C. The lower panel shows the residuals as a function of the independent variable, radius, for a fit to Equation 4 using nonlinear least-squares analysis.

![Figure 2](image2.png)

**FIG. 2.** Apparent weight average molecular weights for OTCase, arginase, and the multienzyme complex versus concentration. Primary data from three different sedimentation equilibrium experiments were transformed by calculating the average molecular weight at each radial position, and pairing that to the appropriate total protein concentration in the centrifuge cell. ■, arginase; ▲, OTCase; ○, multienzyme complex.

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2 R. Tate, H. Schultz, and J. C. Osborne, Jr., unpublished observation.
Fig. 3. Yeast OTCase-arginase multienzyme complex after negative staining. A, negatively stained complex in 1% ammonium formate using double carbon layer technique as described under “Experimental Procedures.” The magnification factor is × 300,000. Panels B–G show enlarged individual molecules of the complex. The magnification factor for these photographs is × 660,000.

| Preparation          | No. of molecules | x axis (Å)       | y axis (Å)       |
|----------------------|------------------|------------------|------------------|
| Negative stain       | 25               | 101.25 ± 3.82    | 84.97 ± 4.96     |
| Rotary shadow        | 25               | 101.81 ± 5.32    | 79.54 ± 5.39     |
| Unidirectional shadow| 25               | 101.81 ± 5.32    | 79.54 ± 5.39     |
| Average              |                  | 102              | 81               |

* Designated as the long axis (width).
* Designated as the short axis (height).
* Rounded to nearest integer.

TABLE I
Molecular dimensions of the OTCase-arginase multienzyme complex
Dimensions are the mean ± 2 S.D.

Sedimentation. Sedimentation was carried out in the presence of 50 mM ornithine and 10 mM agmatine. Agmatine is a competitive inhibitor of arginase and was used in place of arginine to circumvent problems that would arise from the hydrolysis of arginine during the course of the experiment. The primary data from this experiment in the form of total absorbance versus radial position in the ultracentrifuge cell are shown in Fig. 1. Analysis of this data in terms of Equation 4, employing nonlinear least-squares methodology (Johnson et al., 1976, 1981), yields a dissociation constant for complex formation of 2.3 × 10⁻⁹ M. The upper panel shows the primary data and the lower panel shows the distribution of residuals which are small and random. This constant compares with a value of 0.5 × 10⁻⁹ M determined by Penninckx and Wiame (1976) from a steady-state kinetics analysis of OTCase inhibition under similar solvent conditions. The 40-fold difference in the dissociation constants may result from the use of different active-site ligands (ornithine, carbamoyl phosphate, and arginine in the latter case) or from the different methodologies employed to analyze complex formation. The effects that various ligands and combinations of ligands have on the strength of the enzyme-enzyme interaction is currently being investigated.

The association of OTCase and arginase to form the complex may be shown phenomenologically by plotting the apparent weight average molecular weight as a function of total protein concentration. As shown in Fig. 2, the apparent weight average molecular weight of the complex starts to decrease at low protein concentration, indicative of a dissociating system. Also shown are the apparent molecular weights for OTCase and arginase versus total protein concentration as determined in separate sedimentation experiments. Even in the same buffer and in the presence of the effector ligands which promote multienzyme complex formation, the individual enzymes neither dimerize nor aggregate. This experiment provides support for the previous conclusions of Penninckx and Wiame (1976) that, in the presence of active-site ligands for both OTCase and arginase, the two enzymes associate strongly, and establishes the absolute macromolecular stoichiometry of one molecule of arginase associating with one molecule of OTCase.

Since the multienzyme complex could be demonstrated directly using equilibrium sedimentation, additional experiments were performed with various combinations of ligands to evaluate effectors necessary to trigger complex formation. Preliminary experiments (data not shown) suggest that ornithine and ornithine analogs are sufficient to trigger complex formation. Alternatively, phosphate ligands substantially inhibit complex formation.

Electron Microscopy—Sedimentation experiments have demonstrated that a one-to-one complex of OTCase and arginase forms in the presence of their active-site ligands. Since these enzyme are both homologous trimers of known quaternary structure (Duong et al., 1986), it was of interest to determine the architecture of the assembled complex. To
begin to characterize the quaternary structure, it is important to know the dimensions of the complex and, if the resolution is sufficient, to know whether the two trimers exist in a relative staggered or eclipsed configuration. To address these points, electron microscopy of the multienzyme complex was undertaken.

The working field for the multienzyme complex (Fig. 3) is clearly distinct for that of either of the individual enzymes, despite preparation under identical conditions. At low magnification ($\times 300,000$), the field for the complex (panel A) shows a large number of dimeric species. Given in the panels B–G at higher magnification ($\times 660,000$) are pictures of representatives molecules in the dimeric state. Although the trimeric structures of OTCase and arginase are barely apparent at this angle, there is a clear junction delineating the two enzyme molecules. These are evidently views of the complex as seen from the side. Direct measurements on these negatively stained assemblies yielded a width (long axis) of 101.25 Å, and a height (short axis) of 84.97 Å (Table I).

An independent determination of the dimensions of the complex may be obtained from analysis of the complex after rotary shadowing (Fig. 4). Panel A shows a working field ($\times 300,000$) of rotary shadowed complexes. Panels B–G show more highly magnified ($\times 660,000$) examples of individual molecules. The complex prepared in this way is very similar in appearance to the individual molecules after negative staining, although of slightly less resolution. The width and height of the complex determined by this method yield values of 101.81 and 79.54 Å, respectively (Table I). The height of the complex may also be determined from unidirectional shadowing (Fig. 5). Panel A shows a working field of unidirectionally shadowed molecules of $\times 300,000$ magnification. Panels B–G ($\times 660,000$) show enlarged individual molecules of the complex. Here, the shadow is clearly longer than for individual species of the separate molecules prepared in an identical manner (Duong et al., 1986). This technique yields an independent value for the height of 79.42 Å. These values may then be averaged to give an overall width of 102 Å and an overall height of 81 Å (see Table I).

These values are in excellent agreement with the measurements for highly purified OTCase and arginase (Duong et al., 1986). The widths of OTCase (102 Å) and arginase (99 Å) are close to that measured for the complex, and the sum of the heights of OTCase (39 Å) and arginase (42 Å) is identical to the average height of the multienzyme complex. Thus, given the stoichiometry of enzymes in the complex and the molecular dimensions of the enzymes and the multienzyme complex, the architecture of OTCase and arginase in the multienzyme complex suggests a face-to-face orientation. This structure is summarized in Fig. 6. Panel A shows a negatively stained molecule of the complex. Panels B and C show three-dimensional interpretations of the complex shown side-on and from an oblique angle. These were constructed from the dimensions presented here (Table I) and from those of the individual enzymes (Duong et al., 1986). Here the complex is visualized in an eclipsed configuration. However, this choice was arbitrary (see below).

The resolution from simple negatively stained electron micrographs of the complex were insufficient to ascertain the relative orientation of the two enzymes to one another (staggered versus eclipsed) in the assembly. An attempt to resolve this question was made by tilting the sample from +60° to −60° relative to the original normal axis of the support. However, these results (data not shown) were also of insufficient resolution to resolve unambiguously this problem. Hence, a satisfactory answer to this question awaits x-ray diffraction studies.

**Enzyme Kinetics**—The demonstration that OTCase and arginase associate strongly in a one-to-one complex has been shown conclusively using direct methods. However, it is im-
Fig. 5. Yeast OTCase-arginase multienzyme complex after unidirectional shadowing. A, working field of the complex after unidirectional shadowing at a 10° angle. The magnification factor is × 330,000. Panels B-G are of individual dimeric molecules of the complex. The magnification factor is × 660,000.

Fig. 6. Computer-generated models of the OTCase-arginase multienzyme complex. A is an electron micrograph of a negatively stained molecule of the OTCase-arginase multienzyme complex taken from Fig. 1. B and C are computer-generated models of the OTCase-arginase multienzyme complex viewed from above and obliquely.

Fig. 7. Inhibition of OTCase enzyme activity by arginase. An enzymatic titration of 100 ng ml⁻¹ purified OTCase by increasing concentrations of purified arginase. Since the arginase concentration is estimated from activity measurements, the abscissa is given in arbitrary concentration units. Present in the final assay volume of 0.5 ml were 50 μg ml⁻¹ urease, 50 mM ornithine, 2 mM carbamoyl phosphate, 20 mM HEPES, pH 8.0.

Important to establish that OTCase enzyme activity will be inhibited in the presence of its substrates and arginase, as these enzymes were purified from plasmid-containing, enzyme-overproducing yeast strains (Eisenstein et al., 1984; Eisenstein, 1985) with protease-deficient backgrounds. It is possible that an essential post-translational modification
may, or may not, have efficiently been carried out due to the increased biosynthesis of these proteins. Hence, both physical and functional demonstrations of complex formation are essential. As shown in Fig. 7, the enzyme activity of OTCase is inhibited by increasing concentrations of arginase. The theoretical curve describing the data is a simple binding isotherm in which OTCase activity is virtually completely eliminated at high arginase concentration. Moreover, the inhibition of OTCase activity is specific: bovine serum albumin or urease do not inhibit OTCase enzyme activity in kinetics assays. OTCase enzyme activity is reduced only in the presence of arginase.

In conclusion, the regulatory multienzyme complex formed between OTCase and arginase from \textit{S. cerevisiae} has been demonstrated using direct, physical measurements, in addition to being confirmed functionally. These experiments establish the macromolecular stoichiometry of one molecule of arginase to one molecule of OTCase in the complex. This evidence provides support for the proposal that active-site ligands trigger the formation of a tight complex between the two enzymes (Penninckx and Wiame, 1976; Eisenstein and Hensley, 1986). Hence, linkage relationships between subunit interactions in the multienzyme complex and the inhibition of OTCase enzyme activity may now be considered in more detail.

Acknowledgments—We gratefully acknowledge Dr. Francine Messenguy and Professor Jean-Marie Wiame for generously making available enzyme overproducing yeast strains essential to this work and Richard J. Feldmann for constructing the computer-generated models.

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