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Effects of Self-Association of Ornithine Aminotransferase on Its Physicochemical Characteristics†

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ABSTRACT: Previous work in this laboratory [e.g., Peraino, C., Bunville, L. G., & Tahmisian, T. N. (1969) *J. Biol. Chem. 244*, 2241–2249, and Morris, J. E., Peraino, C., & Strayer, D. (1974) *Proc. Soc. Exp. Biol. Med. 147*, 707–709] has shown that the molecular weight of ornithine aminotransferase (OAT) is concentration dependent. In the present study this property of OAT was further characterized by using sedimentation equilibrium centrifugation to determine the molecular weight of OAT in a range of enzyme concentrations. It was shown that OAT aggregates in a two-stage process as its concentration increases. The first stage involves the association of enzymatically active monomers into trimers, with association of the trimers into higher order aggregates occurring in the second stage. Decreasing the pH or raising the ionic strength enhances aggregation, while raising the pH inhibits aggregation; however, the two-stage nature of the aggregation process was not affected by changes in pH and ionic strength. Kinetic analyses of purified enzyme showed that aggregation results in an increase in the K_m for both substrates with the V_{max} remaining constant, indicating that aggregation of monomers sterically hinders substrate binding. Increased K_m values were also obtained for OAT sequestered in mitochondria from rats fed a high-protein diet to increase mitochondrial OAT levels. The higher K_m values suggest that the elevation of OAT in vivo is accompanied by aggregation of the enzyme within the mitochondrion. We propose that the aggregation-dependent increase of K_m in vivo has adaptive value in that it spares ornithine for use in the urea cycle.

Ornithine aminotransferase (EC 2.6.1.13) catalyzes the transfer of the amino group of ornithine to α-ketoglutarate, producing glutamic acid and glutamic semialdehyde. In rat liver, ornithine aminotransferase (OAT)[1] is localized in the mitochondria (Peraino & Pitot, 1963). Although it has been proposed (Herzfeld & Knox, 1968; Volpe et al., 1969) that OAT generates ornithine for use in the urea cycle, considerable evidence (Strecker, 1965; Peraino, 1972; Morris & Peraino, 1976; McGivan et al., 1977) suggests that OAT reduces urea cycle activity by catabolizing ornithine.

Considerable disagreement exists in the reported values for the molecular weight of OAT. Peraino et al. (1969) determined a molecular weight of 132 000. Several investigators (Matsuzawa et al., 1968; Yip & Collins, 1971; Kalita et al., 1976) reported molecular weights from 160 000 to 180 000. Sanada et al. (1976) concluded that there are two forms of OAT: form I with a molecular weight of 177 000 and form II with a molecular weight of 105 000. Evidence that the observed molecular weight of OAT is concentration dependent was presented by Peraino et al. (1969) and Morris et al. (1974), suggesting that OAT undergoes aggregation. In the present study, we have examined the stages of OAT aggregation and assessed the effects of changes in pH and ionic strength on the aggregation process. In addition, we investigated the effects of aggregation on the kinetic properties of the enzyme.

Materials and Methods

Preparation of OAT and Assays of Activity. OAT was purified from rat (*Rattus norvegicus albinus*) liver as described by Peraino et al. (1969) and Morris et al. (1974), except that the pH precipitation step was omitted. Two different procedures were used in assaying the enzyme. One was a single

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[1] Abbreviations used: OAT, ornithine aminotransferase; GDH, glutamate dehydrogenase; EDTA, ethylenediaminetetraacetic acid; NaDodSO$_4$, sodium dodecyl sulfate; OTC, ornithine transcarbamoylase; K_m, Michaelis constant; V_{max}, maximum velocity.

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end point assay described previously (Peraino, 1972; Morris & Peraino, 1976). The second assay involved the continuous measurement of product formation by means of a Gilford 2400-S recording spectrophotometer. This assay is very similar to the single end point assay in that enzyme was added to a cuvette containing both substrates (ornithine and α-ketoglutarate) and the indicator *o*-aminobenzaldehyde. The A_{440} of the adduct formed between the reaction product, Δ^1-pyrroline-5-carboxylate, and *o*-aminobenzaldehyde was then measured continuously. The second assay allowed the measurement of initial reaction rates and was linear with enzyme amount at high enzyme concentrations. OAT concentrations are expressed in activity units per milliliter, where 1 unit is the amount of enzyme that catalyzes the formation of 1.0 μmol of the product/min at 37 °C.

Some experiments utilized a crude enzyme extract prepared as follows. Liver was placed in 2.5 volumes of ice-cold Tris buffer, pH 8.0 (0.01 M Trizma Base, 10^{-4} M pyridoxal 5′-phosphate, 10^{-3} M EDTA, and 0.1% NaN_3; all were from Sigma Chemical Co.). This buffer was used in all subsequent experiments except where noted. Liver was homogenized by using a Polytron PT20 homogenizer. Solubilization of the enzyme was accomplished by freeze–thawing and sonication as described by Peraino et al. (1969) and Morris et al. (1974). The treated homogenate was centrifuged for 45 min at 40000 rpm in a Spinco L-2 ultracentrifuge, and the supernatant containing the enzyme was either stored at −103 °C or used directly.

Molecular Weight Determinations. Molecular weights were determined by using a Beckman air-driven ultracentrifuge (Airfuge) according to the technique developed by Bothwell et al. (1978) and Clarke & Howlett (1979). Centrifugation was performed at 40000 rpm (rotor speed was measured with a stroboscope) for 18 h at room temperature. A standard curve was developed by centrifugation of 10 mg/mL solutions of protein standards of known molecular weights (Pharmacia Fine Chemicals, Inc., and Sigma Chemical Co.). Proteins were dissolved in Tris buffer, and concentrations were determined according to the method of Lowry et al. (1951). The slope ± standard error and *y* intercept of the standard curve (log fraction of protein remaining in the upper third of the Airfuge tube after 18 h of centrifugation plotted on the *y* axis and molecular weight plotted on the *x* axis) are −0.1 ± 0.004 and −0.42, respectively. In Airfuge experiments involving OAT, the amount of enzyme remaining in the upper third of the Airfuge tubes [see Clarke & Howlett (1979)] after 18 h of centrifugation was determined by enzyme assay. The fraction of enzyme in the upper third of the Airfuge tube at equilibrium was used with the standard curve to determine the OAT molecular weight. All aliquots of enzyme from the Airfuge tubes were diluted sufficiently before assay so that linearity was obtained with the single end point assay mentioned above; in this assay the concentrations of both substrates were in high excess. This dilution yielded a final enzyme concentration of ~1.0 μg/mL in the reaction mixture. At this concentration, the enzyme was essentially in its monomeric form (see Results and Discussion).

Determination of Effect of OAT Concentration on Molecular Weight. A solution of purified enzyme in Tris buffer (5.0–11.0 mg of protein/mL) was diluted to provide samples with various OAT concentrations. The specific activities of the enzyme preparations used ranged from 12.0 to 16.0 activity units/mg of protein. In the experiments involving crude OAT preparations, a series of OAT concentrations was produced by diluting the crude supernatant with the Tris buffer. To prevent loss of activity during centrifugation of very dilute solutions of purified enzyme, we maintained the total protein concentration of all samples ~10 mg/mL by adding bovine serum albumin. Preliminary studies indicated that bovine serum albumin had no effect on OAT sedimentation.

Determination of Effects of Ionic Strength and pH on Aggregation of OAT. For studies of the effect of ionic strength on OAT aggregation, KCl was added to OAT to obtain a final KCl concentration of 0.1 M. A range of OAT concentrations was prepared by diluting with Tris buffer that had a KCl concentration of 0.1 M. The effects of pH on aggregation of OAT were studied by using the buffers of Good et al. (1966) to control pH. Approximately 1.0 mL of purified OAT was dialyzed overnight at 2 °C against 4.0 L of a 0.01 M solution of the appropriate buffer [2-(*N*-morpholino)ethanesulfonic acid for pH 6.0 and [tris(hydroxymethyl)methyl]aminopropanesulfonic acid for pH 9.0; both from Calbiochem, Los Angeles, CA] containing pyridoxal 5′-phosphate, EDTA, and NaN_3 at the same concentrations as in the Tris buffer previously described. These preparations were then diluted with the appropriate buffer to obtain a range of OAT concentrations.

Kinetic Studies. The maximum velocity (V_{max}) of OAT and the apparent Michaelis constants (K_m) for both ornithine and α-ketoglutarate were determined by using purified enzymes. Product formation was monitored continuously as indicated above. Kinetic experiments were also performed on OAT sequestered in mitochondria to compare in situ kinetic parameters to those of purified enzyme. Mitochondrial preparations were obtained from two groups of rats as described by Chappell & Hansford (1972). In order to induce an increase in the mitochondrial OAT concentration, we placed rats on a diet of 85% casein (Teklad Mills, Madison, WI) for 2 days before they were sacrificed; for comparison, other rats were fed a standard diet (Wayne Lab Blox, Allied Mills, Chicago, IL). In the experiments using mitochondrial preparations, single end point assays for product formation were done with a Gilford 300-N Micro-sample spectrophotometer. K_m's and V_{max}'s were estimated by using the double-reciprocal plot (Lineweaver & Burk, 1934). Since some investigators (Wilkinson, 1961; Dowd & Riggs, 1965) showed that the double-reciprocal plot is an unreliable method for estimating K_m and V_{max}, the direct linear plot (Eisenthal & Cornish-Bowden, 1974) was also used to estimate these kinetic parameters.

Development of Models to Simulate Aggregation. A two-stage model was developed to predict the relationship between molecular weight and enzyme concentration. The expected concentration-dependent molecular weight of OAT was calculated by assuming the initial aggregation of monomers to form a trimer, followed by aggregation to higher molecular weight oligomers (see Results and Discussion). Trimer formation was characterized by an association constant K_1. As in other multimeric protein assemblies, such as the octameric protein hemerythrin (Langerman & Klotz, 1969), definition of an equilibrium constant for the *n*-mer does not imply that all subunits "simultaneously" interact to form the *n*-mer but may reflect that equilibrium populations of incomplete assemblies are negligible. Accordingly, K_1 is implicitly given by the product $K_{ii}K_{iii}$ where K_{ii} is the association constant for dimer formation, K_{iii} is the constant for dimer–trimer equilibrium, and $K_{ii} \ll K_{iii}$. Association of two trimers is governed by an association constant K_2. For simplicity, it was assumed that further addition of trimeric units was also governed by the same association constant K_2, i.e.

$$M + M + M \underset{K_1}{\rightleftharpoons} M_3 \underset{K_2}{\rightleftharpoons} M_6 \underset{K_2}{\rightleftharpoons} M_9 \ldots$$

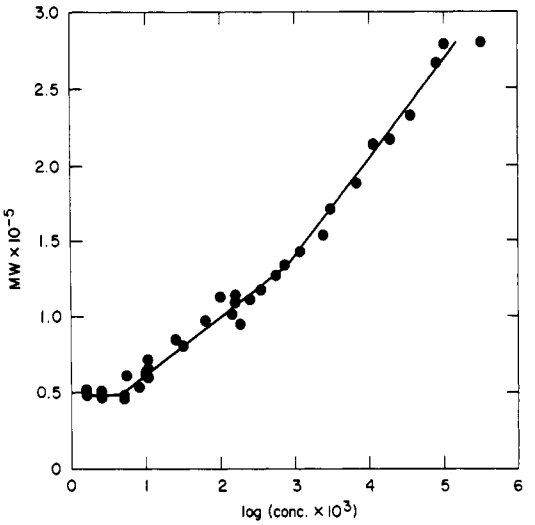

FIGURE 1: Dependence of molecular weight of OAT on enzyme concentration at pH 8.0. Enzyme preparations and determinations of enzyme concentration and molecular weight are described under Materials and Methods. Enzyme concentration is expressed as activity units per milliliter. Each point represents one determination. The lines with positive slopes represent least-squares regression lines. Computerized "break point" analysis (developed by biostatisticians, Division of Biological and Medical Research, Argonne National Laboratory) verified that the regression lines intersect at a molecular weight between 130 000 and 140 000. The slopes of the regression lines ± standard errors are 3.6 ± 0.22 for molecular weights < 140 000 and 6.6 ± 0.26 for molecular weights > 140 000. The slopes are significantly different ($p < 0.01$).

If μ is the free monomer (M) concentration and μ_n is the n-mer concentration, then at equilibrium

$$\mu_3 = K_1\mu^3$$

$$\mu_6 = K_2\mu_3^2 = K_2(K_1\mu^3)^2$$

$$\mu_9 = K_2\mu_6\mu_3 = K_2^2(K_1\mu^3)^3$$

The total monomer concentration is

$$\mu_0 = \mu + 3\mu_3 + 6\mu_6 + 9\mu_9 + \ldots$$
$$= \mu + 3K_1\mu^3 + 6K_2(K_1\mu^3)^2 + 9K_2^2(K_1\mu^3)^3 + \ldots$$

In sedimentation equilibrium, the observed parameter for a reversibly associating system is the weight-average molecular weight (M_w), which is given by

$$M_w = M_1 \frac{\mu + 3^2\mu_3 + 6^2\mu_6 + 9^2\mu_9}{\mu + 3\mu_3 + 6\mu_6 + 9\mu_9}$$
$$= M_1 \frac{\mu + 9K_1\mu^3 + 36K_2(K_1\mu^3)^2 + 81K_2^2(K_1\mu^3)^3}{\mu + 3K_1\mu^3 + 6K_2(K_1\mu^3)^2 + 9K_2^2(K_1\mu^3)^3} \quad (1)$$

where M_1 is the monomer molecular weight and where it is assumed that contributions by oligomers larger than the nonomer are negligible at the protein concentrations used. A computer program was used to calculate M_w for various μ, K_1, and K_2.

For comparison, an elementary one-stage aggregation model based on sequential addition of monomers was examined. The model required that all association reactions be governed by the same equilibrium constant:

$$M + M \underset{K}{\rightleftharpoons} M_2 \underset{K}{\rightleftharpoons} M_3 \underset{K}{\rightleftharpoons} M_4 \underset{K}{\rightleftharpoons} \ldots$$

The weight-average molecular weight in this case is given by

$$M_w = M_1(\mu + 4K\mu^2 + 9K^2\mu^3 + 16K^3\mu^4 + 25K^4\mu^5 + 36K^5\mu^6)/(\mu + 2K\mu^2 + 3K^2\mu^3 + 4K^3\mu^4 + 5K^4\mu^5 + 6K^5\mu^6) \quad (2)$$

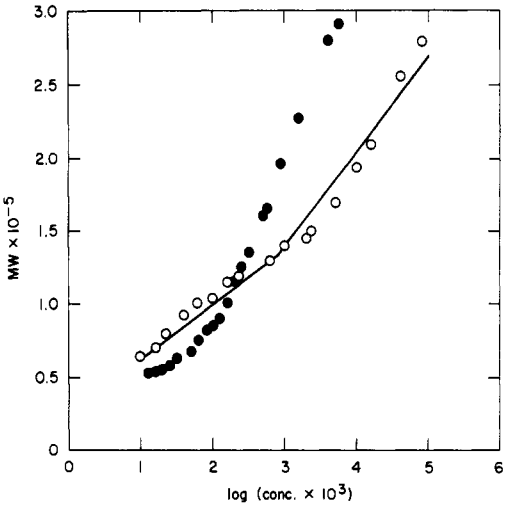

FIGURE 2: Comparison of computer simulation of aggregation of OAT by using two-stage and one-stage models (see Materials and Methods). The open circles represent data points generated by the two-stage model using the association constants listed in the pH 8.0 column in Table I. The closed circles represent data points generated by the one-stage model using the association constant $K = 4.0 \times 10^6\ M^{-1}$. The lines are the least-squares regression lines of the emperical data from Figure 1. Enzyme concentration is expressed as activity units per milliliter.

Results and Discussion

Relationship between Concentration of OAT and Apparent Molecular Weight. The observed dependence of the apparent molecular weight on enzyme concentration (Figure 1) demonstrates the occurrence of aggregation. Aggregation of OAT, which has not been previously characterized, may account for the variability in the reported molecular weight of the enzyme. Although Kalita et al. (1976) reported that the molecular weight of OAT is not concentration dependent, our current data confirm the previous suggestions (Peraino et al., 1969; Morris et al., 1974) that concentration-dependent aggregation of OAT does occur.

Some characteristics of the aggregation of OAT in concentration range between 10^{-3} and 10^3 units/mL can be seen in Figure 1. A molecular weight of 45 000–50 000 is observed at all enzyme concentrations below 4.0×10^{-3} units/mL (0.3 μg/mL). This species apparently represents the monomeric form of the enzyme, which is catalytically active. The 45 000–50 000 molecular weight of the monomer agrees well with published values determined by using $NaDodSO_4$ gel electrophoresis and previously designated OAT subunit molecular weights (Morris et al., 1974; Kalita et al., 1976; Sanada et al., 1976; Lyons & Pitot, 1976). As the enzyme concentration increases, a two-stage process of aggregation appears to occur. The first stage is the formation of trimers with a molecular weight of 130 000–140 000. As the enzyme concentration increases above 1.0 unit/mL, aggregation of trimers appears to occur resulting in complexes with molecular weights >140 000.

Figure 2 depicts computer-generated data points from both the two-stage and one-stage aggregation models. As Figure 2 shows, the two-stage model approximates the empirical data (Figure 1) much better than the one-stage model. The two association constants used in the two-stage model that gave the best fit of the data derived from aggregation studies at pH 8.0 (Figure 1) are listed in Table I. The two-stage model was used in all subsequent aggregation studies to estimate association constants.

The OAT aggregation phenomenon is different from subunit assembly exhibited by most multisubunit enzymes. Friedman

Table I: Effect of pH and Ionic Strength on Association Constants[a] for Aggregation of OAT

	pH 6.0	pH 8.0	pH 9.0	0.1 M KCl
K_1 (M^{-2})	1.0×10^{15}	4.0×10^{14}	8.0×10^{13}	1.0×10^{16}
K_2 (M^{-1})	6.0×10^{4}	6.0×10^{4}	2.0×10^{4}	3.0×10^{5}

[a] The two-stage aggregation model (see Materials and Methods) was used to estimate the association constants.

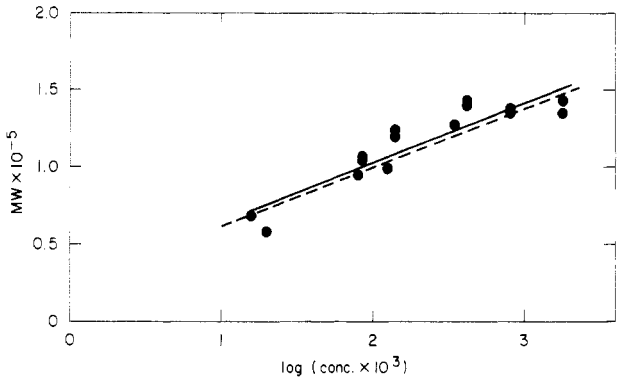

FIGURE 3: Aggregation of OAT in a crude extract at pH 8.0. Preparation of the crude extract and determinations of enzyme concentrations and molecular weights are described under Materials and Methods. Enzyme concentration is expressed as activity units per milliliter. The closed circles represent individual determinations. The solid line is the least-squares regression line of the data (slope ± standard error = 3.8 ± 0.39). The broken line is the least-squares regression line at molecular weights < 140 000 from Figure 1. The slopes of these two regression lines are not significantly different.

& Beychok (1979) reported that studies of protein assembly have generally involved observation of recovery of biological and/or physical properties after inducing disassembly via denaturing agents. OAT differs in that the aggregation of monomers to form oligomers is concentration dependent and reversible, and biological activity is retained in all aggregation states observed (monomers of molecular weight 45 000–50 000 up to and including hexamers of molecular weight 280 000).

Glutamic dehydrogenase (GDH) is an enzyme that exhibits reversible self-association, aggregating in vivo at high concentrations (Olson & Anfinsen, 1952; Frieden, 1958; Kubo et al., 1959). As is the case with OAT, GDH retains its activity in higher aggregation states (Fisher et al., 1962); however, unlike OAT, the active form of GDH is an oligomer with a molecular weight of 316 000 that can be dissociated into identical, inactive subunits with molecular weights of 53 500 (Eisenberg & Tomkins, 1968). At high concentrations the GDH oligomer exhibits reversible, linear, end to end aggregation to form rods of indefinite length (Eisenberg & Tomkins, 1968). Published electron micrographs of polymerized OAT (Morris et al., 1974) indicate that OAT also forms linear rods that are quite similar to GDH polymers in published electron micrographs (Eisenberg, 1971; Josephs, 1971).

The aggregation of OAT in the crude extract is depicted in Figure 3. The slope of the least-squares regression line of these data is not statistically different from the slope of the regression line through the data points depicting trimer formation in a pure enzyme preparation (Figure 1). This indicates that the extraneous proteins found in a crude extract do not affect the aggregation of OAT.

Effect of pH on Aggregation of OAT. The data in Figure 4 indicate that changes in pH do not alter the two-stage nature of the aggregation process. At pH values of 6.0 and 9.0 (Figure 4) and 8.0 (Figure 1), the two regression lines through the data intersect at the trimer molecular weight (130 000–140 000). Changing the pH does alter the association constants

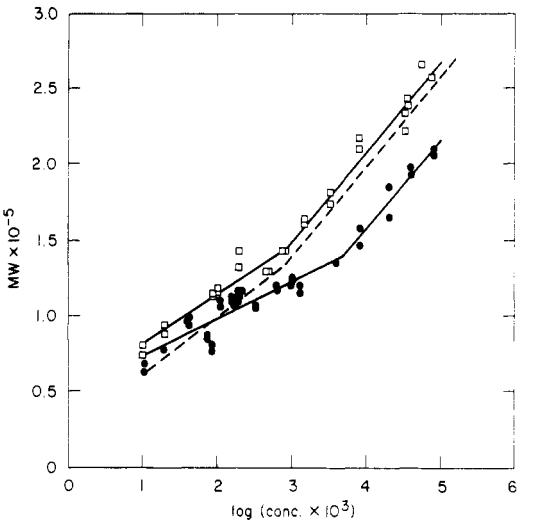

FIGURE 4: The effect of pH on aggregation of OAT (□, pH 6.0; ●, pH 9.0). Preparation of enzyme solutions and concentration and molecular weight determinations are described under Materials and Methods. Enzyme concentration is expressed as activity units per milliliter. The solid lines are least-squares regression lines. Break point analysis verified that at both pH 6.0 and 9.0 the regression lines intersect at molecular weights between 130 000 and 140 000. At pH 6.0, the slopes ± standard errors of the regression lines are 3.4 ± 0.3 for molecular weights < 140 000 and 5.7 ± 0.4 for molecular weights > 140 000. These slopes are significantly different ($p < 0.01$). At pH 9.0, the slopes ± standard errors of the regression lines are 2.47 ± 0.3 for molecular weights < 140 000 and 5.7 ± 0.4 for molecular weight > 140 000. These slopes are also significantly different ($p < 0.01$). The broken lines are the regression lines from Figure 1 (OAT at pH 8.0).

for aggregation, however. As Table I indicates, at pH 9.0 both association constants for OAT aggregation are lower ($K_1 = 8.0 \times 10^{13}$ M^{-2} for trimer formation and $K_2 = 2.0 \times 10^{4}$ M^{-1} for formation of higher aggregation states) than at pH 8.0 ($K_1 = 4.0 \times 10^{14}$ M^{-2} and $K_2 = 6.0 \times 10^{4}$ M^{-1}). Raising the pH apparently inhibits aggregation of OAT. However, decreasing the pH to 6.0 significantly changes only the association constant for trimer formation (as Table I indicates, $K_1 = 1.0 \times 10^{15}$ M^{-2} at pH 6.0, while $K_1 = 4.0 \times 10^{14}$ M^{-2} at pH 8.0). The tendency for aggregation to the trimeric form is increased by decreasing the pH. The isoelectric pH for OAT is 5.4 (Peraino et al., 1969). The increased aggregation of OAT with decreased pH is consistent with the general observation that protein solubility decreases near the isoelectric point.

Effect of Ionic Strength on Aggregation of OAT. A two-stage aggregation process (Figure 5) exists even in the presence of 0.1 M KCl. Again the regression lines intersect at a molecular weight of 130 000–140 000. The association constants for aggregation in the presence of 0.1 M KCl are significantly higher than those for aggregation at lower ionic strength (see Table I). In the presence of 0.1 M KCl, $K_1 = 1.0 \times 10^{16}$ M^{-2} and $K_2 = 3.0 \times 10^{5}$ M^{-1}, while $K_1 = 4.0 \times 10^{14}$ M^{-2} and $K_2 = 6.0 \times 10^{4}$ M^{-1} in the absence of 0.1 M KCl. Thus, increasing the ionic strength increases the tendency for the monomers to aggregate.

Effect of Aggregation on Kinetic Parameters of OAT. Values for K_m and V_{max} were determined for pure enzyme preparations and suspensions of mitochondria. Table II summarizes the apparent K_m values for ornithine and α-ketoglutarate at various aggregation states of purified enzyme. These data show an increase in the K_m values for both substrates as the apparent molecular weight of the enzyme increases. The V_{max} values, however, do not change when normalized for the amount of enzyme in the reaction vessel. The mean V_{max} standard error for the α-ketoglutarate data

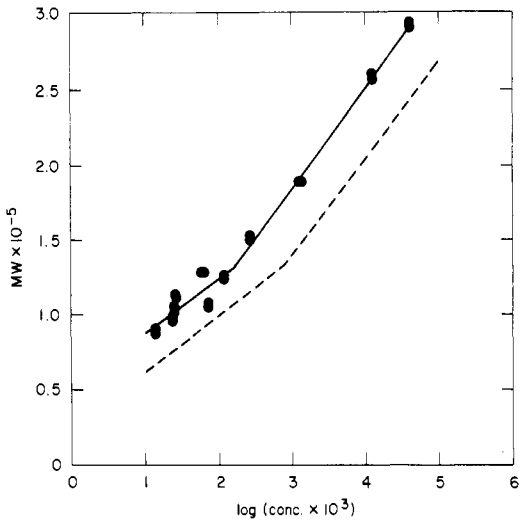

FIGURE 5: The effect of increased ionic strength on aggregation of OAT. Preparation of enzyme solutions and concentration and molecular weight determinations are described under Materials and Methods. Enzyme concentration is expressed as activity units per milliliter. The closed circles represent individual determinations. The solid lines are least-squares regression lines. Break point analysis verified that the least-squares regression lines intersect at a molecular weight between 130 000 and 140 000. The slopes ± standard errors of these regression lines are 3.5 ± 0.7 for molecular weights < 140 000 and 6.7 ± 0.2 for molecular weights > 140 000. The slopes are significantly different ($p < 0.01$). The broken lines are the least-squares regression lines of the data from Figure 1.

Table II: Effect of Aggregation of OAT[a] on K_m Values for Ornithine and α-Ketoglutarate

α-ketoglutarate			ornithine		
	K_m (mM)			K_m (mM)	
OAT M_r	L-B[b]	D-L[c]	OAT M_r	L-B[b]	D-L[c]
160 000	2.1	2.3	160 000	2.8	4.0
135 000	1.7	1.9	130 000	1.3-2.0	2.0-2.3
125 000	1.3	1.4	113 000	1.4	1.6
120 000	0.9	1.0	102 000	1.5	2.8
110 000	1.2	1.2	97 000	1.4	1.2
95 000	1.0	1.0	75 000	1.0	0.9
75 000	0.6-0.9	0.8	64 000	0.7	0.7
65 000		1.0-1.2			
50 000	0.6-0.7				

[a] Purified enzyme at 37 °C; see Materials and Methods. [b] Apparent K_m estimated by using double-reciprocal plot; see Materials and Methods. [c] Apparent K_m estimated by using direct-linear plot; see Materials and Methods.

is 0.048 ± 0.001 absorbance units s^{-1} (activity unit)$^{-1}$, while the mean V_{max} standard error for the ornithine data is 0.047 ± 0.001 absorbance units s^{-1} (activity unit)$^{-1}$. The aggregation-dependent increase in K_m values coupled with the constant V_{max} suggest competitive inhibition. Therefore, the decrease in the enzyme–substrate interaction as enzyme concentration increases is evidently due to the production of steric hindrance at the active sites by the association of the monomers.

The observations that aggregation of OAT in vitro results in increases in the K_m values for α-ketoglutarate and ornithine suggested that K_m values might be used to detect in vivo aggregation of the enzyme. The previous observation (Peraino, 1967) that high-protein diets induce increased levels of OAT was exploited to obtain mitochondrial-sequestered enzyme at low and high in vivo concentrations. Mitochondrial suspensions from rats fed an 85% casein diet or a 24% protein control diet were prepared, and α-ketoglutarate K_m values were determined; the results are presented in Table III. The data show

Table III: α-Ketoglutarate K_m Values of Mitochondrial-Sequestered OAT

		α-ketoglutarate K_m[a] (mM)	
diet[b]	enzyme activity[c]	mitochondrial suspension	sonicated samples
24% protein	0.52 ± 0.1	0.60 ± 0.07 (4)	0.69 ± 0.04 (6)
85% casein	0.94 ± 0.01	0.94 ± 0.07 (11)	0.64 ± 0.04 (7)

[a] Mean ± standard error (number of determinations). [b] See Materials and Methods for a description of diets. [c] Activity units per milliliter ± standard error (see Materials and Methods for definition of activity units of OAT).

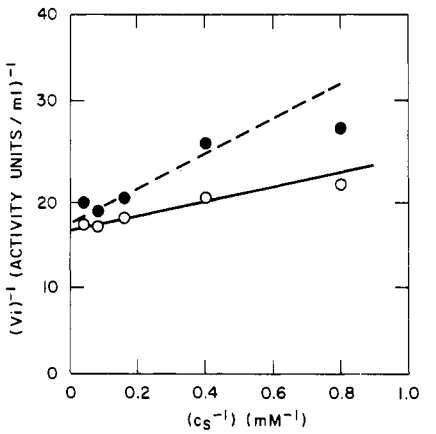

FIGURE 6: Lineweaver–Burk plots for OAT sequestered in mitochondria. Preparation of mitochondria and initial velocity (V_i) determinations are described under Materials and Methods. C_s represents α-ketoglutarate concentration. The closed circles represent data from mitochondrial suspensions obtained from rats fed an 85% casein diet. The open circles represent data from mitochondrial suspensions obtained from rats fed a 24% protein diet (control).

that the α-ketoglutarate K_m of the mitochondrial OAT was significantly increased ($p < 0.05$) by feeding rats an 85% casein diet for 2 days.

The K_m for ornithine in mitochondrial-sequestered OAT was not determined because prior studies (McGivan et al., 1977) suggest that the transport of ornithine into mitochondria is rate limiting, while α-ketoglutarate transport is not rate limiting (Palmieri et al., 1972). However, in view of the OAT concentration-dependent increases in the α-ketoglutarate K_m in vivo as well as in vitro, the assumption that the ornithine K_m increases in vivo as well as in vitro appears reasonable.

To test the hypothesis that the increase in the K_m of the mitochondrial enzyme from rats fed the 85% casein diet is the result of in vivo aggregation, we sonicated the mitochondrial suspensions from the two treatment groups to release the enzyme and determined the K_m's. As shown in Table III, sonication of the control sample had no significant effect on the K_m for α-ketoglutarate; in contrast, disruption of the mitochondria from the animals fed the 85% casein diet resulted in the decrease of the K_m to a level similar to that of the control. The latter values are similar to α-ketoglutarate K_m values of the purified monomeric enzyme (see Table II). These results support the hypothesis that sufficiently increasing the mitochondrial concentration of the enzyme in vivo results in aggregation of OAT monomers. When mitochondria containing aggregated OAT monomers are sonicated, the enzyme aggregates are released into a much larger volume and dissociate to monomers.

Further characterization of the in vivo aggregation of OAT may be obtained by comparison of Lineweaver–Burk plots derived for the two intact mitochondrial suspensions (Figure

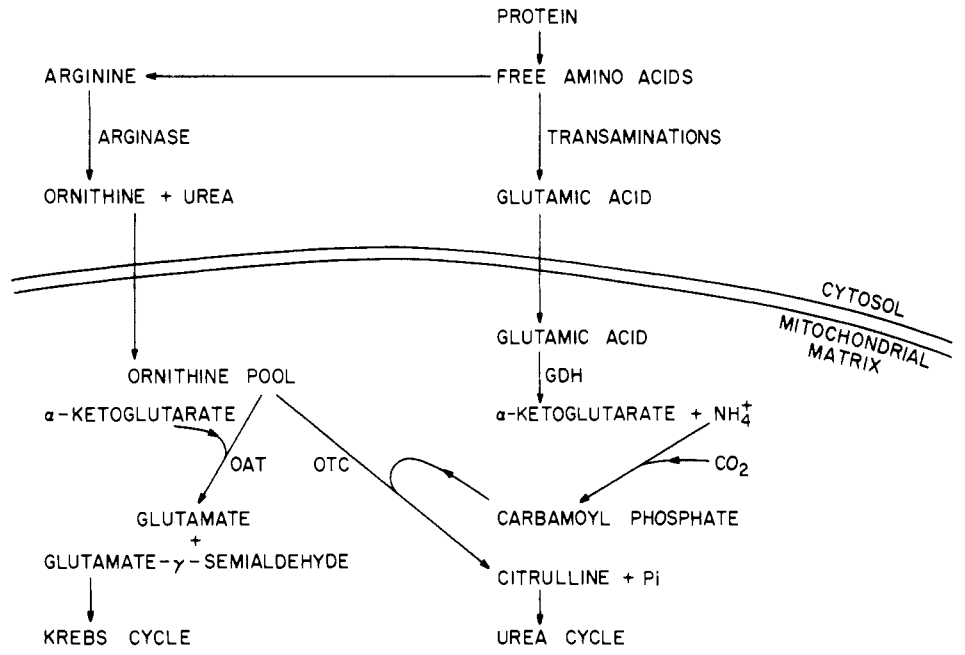

FIGURE 7: The relationship between OAT, the urea cycle, and the Krebs cycle. Abbreviations: OAT, ornithine aminotransferase; OTC, ornithine transcarbamyolase; GDH, glutamate dehydrogenase.

6). The mitochondrial suspensions from rats fed an 85% casein diet were diluted until the total activity present was approximately equal to that in the control. The Lineweaver–Burk plots in Figure 6 indicate the presence of competitive inhibition, i.e., the plots have the same $(1/V)$ intercept and different slopes. These observations represent additional evidence that competition exists between substrate binding and monomer association.

Implications of Aggregation of OAT for Metabolic Regulation. Several workers (Strecker, 1965; Peraino, 1972; Morris & Peraino, 1976; MgGivan et al., 1977) have proposed that OAT may regulate the urea cycle by decreasing the ornithine pool in the mitochondria. Figure 7 depicts the relation between OAT, the urea cycle, and the Krebs cycle. According to this scheme, OAT generates glutamate and glutamate semialdehyde for use as Krebs cycle intermediates by utilizing the ornithine pool in the mitochondrion. Thus, OAT competes with the urea cycle enzyme ornithine transcarbamoylase (OTC, EC 2.1.3.3) for available ornithine. Increased OAT activity diminishes the amount of ornithine available for use by OTC and therefore decreases the activity of the urea cycle.

When rats are fed a high-protein diet, OAT levels in the liver are increased (Peraino, 1967). The stimulus is in part an increase in the level of ornithine produced by catabolism of arginine (Civen et al., 1967). Increased OAT activity will facilitate the transfer of carbon skeletons from excess ornithine to the Krebs cycle (Figure 7). These reactions apparently play a role in gluconeogenesis, since glucagon (a hormone that elevates blood glucose) stimulates OAT synthesis in rats fed a low-protein diet (Lyons & Pitot, 1976), and dietary carbohydrate represses the effects of both glucagon and dietary protein in OAT levels in rat liver (Peraino & Pitot, 1964). Thus, induction of OAT synthesis is adaptive in that it allows the animal to respond to elevations in the ornithine pool by increasing the rate of conversion of ornithine to carbohydrate.

However, when rats are placed on a high-protein diet, the urea cycle activity also increases (Schimke, 1962, 1963). As Figure 7 shows, a high-protein diet results in increased levels of NH_4^+ in the liver mitochondria due to transaminations of amino acids and subsequent deamination of glutamic acid. NH_4^+ is toxic and must be converted to urea, which is much less toxic. Under the conditions of a high-protein diet, then, increased levels of OAT activity would seem to be detrimental in that increased OAT activity would tend to deplete the ornithine pool and therefore reduce the ability of OTC to function in the conversion of NH_4^+ to urea.

The data in Table III indicate that rats fed a high-protein diet exhibit in vivo aggregation of OAT. As aggregation occurs, the apparent K_m values for both ornithine and α-ketoglutarate increase (Table II). The decreased enzyme–substrate interactions (due to increased K_m's for both substrates) reduce the catalytic efficiency of the enzyme. This reduced efficiency may ameliorate the increased drain on the ornithine pool that results from OAT synthesis. Thus, aggregation of OAT has physiological significance in that it spares ornithine for use by OTC in converting excess NH_4^+ to urea, even though synthesis of OAT has been stimulated. In this way, OAT retains the ability to increase the rate of conversion of ornithine to carbohydrate, yet aggregation provides a buffering effect on the inhibition of the urea cycle that would otherwise occur as a result of increased synthesis of OAT.

Further evidence that aggregation of OAT is adaptive because it spares ornithine is seen when the ornithine K_m of OTC is compared with the change in the ornithine K_m of OAT as aggregation occurs. Hoogenraad et al. (1980) reported that rat liver OTC exhibits an ornithine K_m of 1.25 mM. Table II indicates that for monomeric OAT the ornithine K_m is <1.0 mM. Monomeric OAT will therefore more readily bind available ornithine than will OTC. However, as Table II shows, when OAT aggregates to form trimers, the ornithine K_m increases to >2.0 mM. Since for trimeric OAT the ornithine K_m is larger than the ornithine K_m for OTC, OTC will more readily bind available ornithine than will OAT trimers. Matsuzawa (1974) reported that the intramitochondrial steady-state ornithine concentration in rat liver was not measurable in the nanomole per gram of mitochondria (wet weight) range. Because the intramitochondrial ornithine concentration is much lower than the ornithine K_m's of OAT and OTC, the differences in the ornithine K_m's of these two enzymes are relevant to our contention that aggregation of OAT is of functional significance because it spares ornithine for use by the urea cycle.

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