Assay and Kinetics of Arginase

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A sensitive colorimetric assay for arginase was developed. Urea produced by arginase was hydrolyzed to ammonia by urease, the ammonia was converted to indophenol, and the absorbance was measured at 570 nm. The assay is useful with low concentrations of arginase (0.5 munit or less than 1 ng rat liver arginase) and with a wide range of arginine concentrations (50 μM to 12.5 mM). Michaelis–Menten kinetics and a K_m for arginine of 1.7 mM were obtained for Mn^{2+}-activated rat liver arginase; the unactivated enzyme did not display linear behavior on double-reciprocal plots. The kinetic data for unactivated arginase indicated either negative cooperativity or two types of active sites on the arginase tetramer with different affinities for arginine. The new assay is particularly well suited for kinetic studies of activated and unactivated arginase. © 1986 Academic Press, Inc.

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Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) is the last enzyme in the urea cycle and catalyzes the hydrolysis of arginine to ornithine and urea. The properties of arginase from various sources have been studied in several laboratories over a period of 40 years; however, there are several discrepancies in the kinetic data reported, even for arginase from a single organ and species. For example, many published estimates of the Michaelis constant (K_m) for arginine with rat liver arginase are in the 10–20 mM range (1,2), but values as low as 1.0 mM (3) have been reported. The variability in arginase kinetic data may be due to the variety of assays used. The assays measure either loss of arginine, formation of either urea or ornithine, or production of ammonia or carbon dioxide by the action of urease on urea. The most frequently used assays measure the loss of arginine or the formation of urea, but all of the assays have problems associated with them. One commonly used assay, the Van Slyke and Archibald assay (4,5) and its subsequent modifications (6,7,8), requires boiling the urea formed by arginase with a color reagent for 1 h in concentrated acid to form the colored product which is also light sensitive. In addition, the assay can only be used with arginine concentrations above 10 mM and requires that so much arginine be hydrolyzed for urea determinations that the ornithine produced is at a potentially inhibitory concentration.

Ward and Srere (9) introduced an assay which directly measured the loss of arginine by monitoring the absorbance at 205 nm. This assay was more rapid and convenient than the Van Slyke and Archibald assay, but could only be used in the range of 0.5–2.0 mM arginine. Pace et al. (3) extended the theoretical range of arginine concentrations to 0.1–12.5 mM arginine by measuring the decrease in absorbance at wavelengths above 205 nm. While the assay can accurately determine initial velocities, it is limited by the fact that manganese, a cofactor, interferes with the assay. In addition, the spectrophotometer best suited for this assay is a very sensitive double-beam instrument with a water circulator to control the reaction temperature; this is not always available, thereby reducing the practical usefulness and sensitivity.

Assays using radiolabeled substrate are very sensitive, as is the assay described by Ruegg.
Arginase was used as the substrate and urea was separated from unhydrolyzed arginine using a sulfonate resin. The assay can detect 0.1 munit of arginase compared to 50 munit measured by colorimetric assays, but cannot be used to measure the time course of urea production without preparing separate reaction mixtures for each of the time points. Also, special care must be taken because of the use of the radio-labeled substrate.

There have been several attempts to measure arginase activity by hydrolyzing the urea produced to ammonia and carbon dioxide with urease. With the urease electrode, the urea was hydrolyzed by the electrode and the ammonia determined potentiometrically using an ammonia-sensing electrode (11). However, the electrodes can be expensive and the assay requires high arginine concentrations. The carbon dioxide produced by urease can also be quantitated by bubbling it through a dilute acid solution and titrating the remaining acid (12). Although this procedure is very accurate, it is not suitable for use on multiple samples.

We report here an assay for arginase where the urea produced by arginase is hydrolyzed to ammonia and carbon dioxide by urease and the ammonia is subsequently converted to indophenol which can be measured colorimetrically (Scheme 1). Sensitivity is approximately 0.5 munit (approximately 1 ng of purified arginase) which is in the same range as assays using $^{14}$C-arginine. In addition to the high sensitivity, the assay is inexpensive, simple, very reproducible, and quite flexible, allowing for wide variation in the concentration of substrate and enzyme. A $K_m$ of 1.7 mM arginine was obtained using the new assay for activated rat liver arginase, as compared to a $K_m$ of 1.3 mM reported by Pace et al. (3). We have also found evidence indicating that unactivated arginase is either made up of subunits with different kinetic parameters or that it exhibits negative cooperativity. It is because of the high sensitivity and the wide range of useful arginine concentrations (greater than 150-fold) of this assay that this previously unreported kinetic behavior of unactivated arginase was observed.

**MATERIALS AND METHODS**

Arginase was purified from the livers of Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.) using a modification of a procedure described by Schimke (13). Following ammonium sulfate precipitation and dialysis, the sample was applied to a Green A-Sepharose column (Ami-

\[ \text{H}_2\text{NCH}_2\text{CH}_2\text{C}_6\text{H}_4\text{COO}^- + \text{NH}_3 \rightarrow \text{NH}_2\text{CH}_2\text{CH}_2\text{C}_6\text{H}_4\text{COO}^- + \text{NH}_3 \]

\[ \text{Guanidino-$}^{14}\text{C}$-arginine $\rightarrow$ guanidino-$^{14}\text{C}$-ornithine $\rightarrow$ ornithine $\rightarrow$ citrulline $\rightarrow$ arginine $\rightarrow$ urea $\rightarrow$ ammonia $\rightarrow$ indophenol

\[ \text{NH}_3 + 2 \text{C} = \text{N} \rightarrow \text{NH}_2\text{CH}_2\text{CH}_2\text{C}_6\text{H}_4\text{OH} \]

\[ \text{Scheme 1.} \]
con Corp., Danvers, Mass.; 1.5 × 7.5 cm) in 10 mM Tris-HCl, pH 7.0, and, after a gradient of 0–0.15 M KCl in the same buffer, arginase was eluted with 0.15 M KCl. The active fractions were pooled, dialyzed against 10 mM Tris-HCl, pH 7.0, and applied to a second Green A column (0.75 × 3.0 cm). The column was washed with 10 mM Tris-HCl, pH 7.0, and the arginase was eluted with 10 mM Tris-HCl/0.15 M KCl, pH 7.0. Protein concentration was determined by the method of Kalb and Bernlohr (14). The purified arginase, when fully activated, had a maximal specific activity of 2000 units/mg using the Van Slyke and Archibald assay; a single protein band of 35,000 Da was evident after electrophoresis on sodium dodecyl sulfate-polyacrylamide gels containing β-mercaptoethanol and staining with silver stain (Bio-Rad, Richmond, Calif.) (15). Arginase (1 mg/ml) was activated by incubation with 50 mM MnCl₂ and 10 mM Tris-HCl, pH 7.0, at 55°C for 10 min. Following activation, arginase was diluted to a concentration appropriate for a particular assay.

To compare the various assays, arginase activity was measured using the direct spectrophotometric assay of Pace et al. (3) and the method of Van Slyke and Archibald (4) which estimates urea produced using α-isonitroso-propiophenone. One unit of arginase activity was defined as the production of 1 μmol urea per minute. For the spectrophotometric assay, the absorbance at 205 nm of a 1.00 ml assay mix was measured on a Beckman Model 34 spectrophotometer. All arginine solutions were adjusted to pH 9.5.

Urease (Type VII) was dissolved in 50 mM sodium acetate, pH 5.0, to yield a final concentration of 15 units/ml. One unit of urease liberates 1.0 μmol ammonia per minute. The color reagents for the conversion of ammonia to indophenol are a modified form of the concentrated reagents described by Chaney and Marbach (16). Briefly, the phenol nitroprusside reagent contained 50 g phenol and 0.50 g sodium nitroprusside per liter and the alkaline hypochlorite solution contained 25 g sodium hydroxide and 42 ml sodium hypochlorite (4–6%, Fisher Scientific Co., Silver Spring, Md.) per liter. The alkaline hypochlorite reagent is stable for at least 1 month, but the phenol nitroprusside should be discarded when the blank exceeds 0.1 (approximately 1 week if it is stored in a dark bottle). All reagents were prepared using water from a Millipore Q filtration system (Millipore Corp., Bedford, Mass.) and had a resistance of 18 MΩ. These reagents are available in kit form (Sigma Chemical Co.); however, the blank is fourfold higher than that of freshly prepared reagents and additional buffer must be added to the buffered urease supplied with the kit to ensure that the pH is below 6.0 after addition of the reaction mixture. This prevents further hydrolysis of arginine because arginase is not active below pH 7.

To assay for arginase activity, the sample to be assayed (usually 50 ng arginase) was incubated with arginine (usually 10 mM was used to follow a purification) and 5 mM sodium bicarbonate, pH 9.5, in a final volume of 1.5 ml at 25°C. Other buffers may be used provided they do not contain primary amino groups which result in very high blanks. Portions (0.25 ml) of the assay mixture were removed at various time points (1 to 5 min intervals) and added to the same volume of buffered urease (see above). After a 30-min incubation with urease, the reaction was stopped by the addition of 1.00 ml of each of the color reagents with mixing after each addition. Absorbance at 570 nm was measured at least 30 min after addition of the color reagents and compared to a standard curve prepared using 0–0.10 μmol urea (Electrophoresis grade, Bio-Rad, Richmond. Calif.) in 0.25 ml containing buffer and arginine at the concentrations present in the assay.

Ammonia production by urease was found to be completed within 30 min at 25°C. The incubation with urease may be shortened by incubating at a higher temperature (up to 37°C) or by increasing the urease concentration. Color development was maximal under the conditions described above after an incubation of at least 30 min with the two color
reagents, and the color was stable for at least 24 h, even when the tubes were kept in a well-lit area. Following the color reaction, absorbance was determined at a wavelength between 570 and 645 nm (the wavelength of maximum absorbance for indophenol), depending on the amount of arginase activity present in the assay. The levels of activity (μmol urea produced/min/mg) were similar if the absorbance was determined at 570 or 645 nm, although low levels of activity were more accurately determined if the absorbance was measured at 645 nm.

Unless otherwise stated, all chemicals were reagent grade and obtained from Sigma Chemical Company, St. Louis, Missouri. The values of the Michaelis constant were determined by linear regression analysis of the particular kinetic plot used.

RESULTS

Arginase activity was found to be directly proportional to the amount of arginase protein present in the range of 0.10 to 1.0 μg/ml assay mixture (Fig. 1). Above 0.14 unit, the high concentration of arginase resulted in the hydrolysis of more than 100% of the arginine over a 4-min time course. Arginase concentrations may exceed 0.14 unit if samples are removed at proportionally shorter intervals; this would ensure that the velocity measured is actually the initial velocity. The assay was also useful in the range of 1 to 100 ng arginase per milliliter of assay mixture and was directly proportional to enzyme concentration in this range.

Several substances were tested for interference with this assay. They include isoleucine, valine, leucine, ornithine, lysine, 2-methylbutyric acid, 3-methylpentanoic acid, 5-aminopentanoic acid, 8-aminooctanoic acid, 2-aminohexanoic acid, and norleucine: none were found to have any effect on the color reaction. Canavanine caused the formation of a green-brown product that resulted in a higher blank, but had no effect on the slope of the standard curve. Arginine did effect the standard curve slightly, causing the slope to decrease linearly with increasing arginine concentrations. This interference was compensated for by including arginine in the standard curve at the same concentration as in the assay. It is this interference which limits the use of this assay to arginine concentrations below 15 mM.

The activity measured using the new assay was compared to the activity measured using the Van Slyke and Archibald and the Pace assays, as seen in Fig. 2. Using the Pace assay and a Beckman Model 34 spectrophotometer, it was possible to measure the activity for arginine concentrations between 0.2 and 2.0 mM, while the Van Slyke and Archibald assay is accurate at arginine concentrations above 10 mM. Using the new assay, we were able to measure the activity in the range of 0.05–12.5 mM arginine which covers the entire range of the Pace assay and extends to the lower limits of the Van Slyke and Archibald assay. Results obtained with unactivated arginase using our assay agreed well with those obtained with both the Pace and the Van Slyke and Archibald assays, although the amount of arginase in our assay was approximately 100-fold less than that necessary for the other two assays.

![Fig. 1. Arginase activity as a function of enzyme concentration. Activity at 25°C was measured using the new assay with unactivated rat liver arginase, 5 mM sodium bicarbonate, and 5 mM arginine at pH 9.5 (1.5 ml total volume). Samples of the incubation mixture were withdrawn at 1-min intervals and urea formed was quantitated as described under Materials and Methods and Scheme 1.](image-url)
FIG. 2. Comparison of initial velocities measured at various arginine concentrations using the Pace et al. (3) direct spectrophotometric assay (○), the Van Slyke and Archibald (4) assay (□), and the assay we described here (▲). For the Pace and Archibald assays, incubation mixtures contained 8 μg unactivated arginase. For the new assay, incubation mixtures contained 95 ng unactivated arginase. The inset shows activities at the lower arginine concentrations. Initial velocities are all expressed as μmol urea produced/min/8 μg in a 1.5-ml reaction mixture.

Similar results were also obtained when activated arginase was used in the Van Slyke and Archibald assay or the new assay (data not shown). Comparisons of the new assay with the Pace assay using activated arginase was not possible because manganese interferes with the latter assay.

The effect of substrate concentration on velocities measured using the new assay with activated and unactivated arginase is shown in Fig. 3 on double-reciprocal plots. With activated arginase, a $K_m$ for arginine of 1.7 mM was obtained and the enzyme displayed Michaelis–Menten kinetics. Linear behavior was not observed for unactivated arginase on double-reciprocal plots; velocities at lower arginine concentrations (below 3 mM) appeared to be higher than would be predicted from the velocities at arginine concentrations above 3 mM. This resulted in a downward curvature on Lineweaver-Burk plots. A Scatchard plot of the data also deviated from linearity (Fig. 4). The shape of both the Lineweaver-Burk and Scatchard curves could indicate either negative cooperativity or that arginase has two different types of active sites. A Hill plot of the kinetic data using unactivated arginase resulted in a straight line with a Hill coefficient of 0.62 compared to a Hill coefficient of 1.08 obtained for activated arginase.

DISCUSSION

Many arginase assays have been described over the past 40 years, but most of these assays were tedious, inconvenient, inappropriate for multiple samples, or had a low sensitivity. The assay described here overcomes these problems. The sensitivity is 100-fold greater than that of the most sensitive previously described

FIG. 3. Lineweaver-Burk analysis of initial velocities measured using the new assay. Assays were performed at 25°C using 95 ng of either unactivated (▲) or activated (◇) arginase. (A) The arginine concentrations range from 0.1 to 12.5 mM; (B) Includes only arginine concentrations from 0.67–17.5 mM.

FIG. 4. Scatchard analysis of initial velocities obtained using the new assay and unactivated arginase. Assay conditions were the same as those in Fig. 2.
ARGINASE ASSAY

For this reason, the apparent $K_m$ is often determined and many different values have been reported. The apparent $K_m$ we have obtained (1.7 mM) for activated rat liver arginase using the new assay agrees with the value obtained by Pace using both the direct spectrophotometric (1.3 mM) and the Van Slyke and Archibald (1.6 mM) assays. We obtained an apparent $K_m$ of 12.5 mM arginine using the Van Slyke and Archibald assay which is in the range of values reported by other investigators using this assay; however, no correction was made for substrate depletion or inhibition by products (3).

Using the assay described here, we were able to observe some interesting nonlinear kinetics with unactivated rat liver arginase. The kinetic data indicated either that there are two types of active sites in the arginase tetramer that act independently or that the enzyme displays negative cooperativity. Using this data, we cannot distinguish between these two possibilities. It has been shown by physicochemical methods that the unactivated enzyme contains two manganese atoms per tetramer (18). Thus, the manganese-binding and manganese-free sites might account for the two sites observed in kinetic data. The independent action of these two types of sites seems feasible since activated arginase does not exhibit negative cooperativity (Hill coefficient = 1.08). It is also possible that the manganese-free sites have no activity and that the two sites containing manganese have different affinities for arginine. Negative cooperativity would also explain the kinetic data and might help to prevent the accumulation of ornithine and/or urea at higher arginine concentrations.

ARGINASE isozymes are often classified according to the value of the Michaelis constant.
which best resemble those seen *in vivo* (20). It is possible that arginase, with its many control mechanisms (activation and $K_m$ much higher than the *in vivo* concentration of substrate and the kinetics we have observed here), plays a role in controlling the rate of the urea formation and arginine degradation.

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**REFERENCES**