Nuclear Magnetic Resonance Studies of Manganese Binding of Rat Liver Arginase

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SUMMARY

Magnetic resonance studies of the activation of arginase (L-arginine amidinohydrolase, EC 3.5.3.1) with manganese have been performed with nuclear magnetic resonance. The results showed that 4 moles of manganese were bound per mole of fully activated arginase. However, the binding affinities of the metal ions to the enzyme were not identical; 50% of the Mn²⁺ was strongly bound and could not be removed without considerable irreversible loss of activity and solubility of the enzyme. Various treatments such as dialysis, electrophoresis, or chelating of the Mn²⁺ by an excess of complexing agents resulted in enzyme preparations that retained about 50% of their original activity; full activity could be recovered in those samples by the addition of Mn²⁺. The amount of tightly bound manganese estimated by quantitative electron spin resonance measurements after denaturation of the enzyme with 1.5 M HCl showed that the dialyzed enzyme, with a residual catalytic activity of 50%, contained 2 moles of manganese per mole of arginase, suggesting that the enzyme existed then in the form of an E-Mn₂ complex. Measurements of the effect of the manganese in the E-Mn₂ complex on the longitudinal nuclear relaxation rate of water protons at pH 7.5 and 34° yielded an enhancement of 8.0 and a binding constant (K₂) whose lower limit is 3 × 10⁶ M⁻¹.

The activation step of arginase from E-Mn₄ → E-Mn₄, which results in a restoration of 100% of the catalytic activity, was studied kinetically at increasing manganese concentrations and at various temperatures and pH. By titration of the enzyme with manganese and vice versa, the formation of the E-Mn₄ complex was followed by measurements of the proton relaxation rate, which yielded an enhancement of 6 to 8 at pH 7.5 and 34°. The binding constant for the reversibly removable Mn²⁺ was calculated to be K₂ = 2 ± 1 × 10⁴ M⁻¹.

The addition of arginine (substrate) or ornithine (competitive inhibitor) did not further increase the enhancement; rather, it slightly lowered it, which proves that primarily a binary complex between arginine and manganese is formed.

divalent metal ions, e.g. Mn²⁺, Co²⁺, Ni²⁺ (1-3) and in some instances Fe²⁺, VO²⁺, and Cd²⁺ (4, 5). It is also well established that the stability of the enzyme is increased by the addition of certain divalent metal ions, particularly Mn²⁺ (6). The nature of the involvement of these cations for the structure and biological activity of arginase has not yet been established. For further elucidation of the enzymatic mechanism, as well as of the characterization of the enzyme, it was of interest to investigate the binding properties of the arginase-manganese complex.

Magnetic resonance techniques offer a sensitive and valuable tool for exploring the interactions between proteins and paramagnetic ions, as has been demonstrated in a number of investigations (7-13).

This paper reports the results of kinetic and magnetic resonance studies of arginase from rat liver with the paramagnetic ion Mn²⁺ as activator. The correlation between catalytic activity and manganese binding, the number of binding sites and the binding constants were determined.

MATERIALS AND METHODS

1. Arginine (free base), L-ornithine, and tris(hydroxymethyl)aminomethane (A grade) were obtained from Calbiochem. MnCl₂ (hydrated), analytical reagent grade, was purchased from Mallinckrodt Chemical Works; isotopic ⁵⁴MnCl₂ from New England Nuclear; bovine serum albumin (crystallized) from Pentex; isonitrosopropiophenone from Eastman Kodak Company, Rochester, New York; and EDTA from J. T. Baker Chemical Company. Diethylenetriamine pentaacetic acid and 2-hydroxyethylthlenediamine triacetic acid were obtained from Koch-Light Laboratories, Inc., London, England. Dithylethanolamine pentaacetic acid and 2-hydroxyethylethylenediamine triacetic acid were obtained from K & K Laboratories, Plainview, New York. Chelating resin (Chelex-100) was purchased from BioRad, Richmond, California.

Rat liver arginase was purified as described previously (14, 15).

The enzyme preparations had a specific activity of 18,000 to 20,000. One enzyme unit was defined as the amount of enzyme that produces 1 μmole of urea per min at 25° (16). Specific activity was expressed in enzyme units per mg of protein nitrogen. For the determination of enzyme activity, either the urea produced was assayed with isonitrosopropiophenone (17) or ornithine was determined in a coupled enzymatic reaction with L-ornithine aminotransferase, glutamic dehydrogenase, and NAD (18).

For all calculations, the molecular weight of the rat liver enzyme was taken as 118,000 (17). Protein determinations were carried out with Folin-Ciocalteu reagent (19), with the use of bovine serum albumin as a standard. Because manganese at a concentration of >0.01 M interferes with the assay, enzyme samples were dialyzed prior to protein determination.

Isoelectric focusing was essentially performed according to a

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method described by Vesterberg and Svensson (20), with the use of carrier ampholytes of LKB-Produkter, Stockholm, to establish a pH gradient of 3 to 10 or 7 to 10. Pure arginase (3.5 mg, approximately 9000 enzyme units) in a volume of 1 ml was applied on the column. The experiments were carried out for a period of 64 hours. The voltage was increased stepwise from 200 to 550 volts and kept constant at the upper figure. The column temperature was 5°. Fractions of 2 ml were collected from the bottom of the column, and enzyme activity, protein concentration, and pH were determined for each fraction. The pH measurements were made at 4° with a Beckman Zeromatic II pH meter, equipped with a combination electrode and an automatic temperature compensator.


tyramine used in the dialysis experiment was prepared by activation of a dialyzed arginase preparation (the enzyme had only 50% of its original activity due to dissociation of manganese) with a 0.05 M MnCl₂ solution containing traces of ⁵⁴Mn. Full activation was achieved by incubation in a 50° water bath for 10 min. Radiactive manganese was counted in a Packard well-type scintillation detector.

The measurements of the proton relaxation rates were carried out with a Varian model A-60A. The amount of free manganese was measured by electron spin resonance spectroscopy at 9.5 GHz using 100 kH, field modulation.

To measure quantitatively the nondialyzable manganese bound by the enzyme, samples of 7.2 to 36 mg of arginase were incubated for 1 to 3 days in 1 ml of 1.5 N HCl in a rotating shaker at room temperature. The samples were then centrifuged for 15 min at 15,000 g, and the precipitate was washed twice with 0.25 ml of 1.5 N HCl. The amount of free manganese in the combined supernatants was determined by ESR (21).

**Magnetic Resonance Measurements**—The enzyme-bound manganese was studied by its effect on the proton relaxation rate of water by the nuclear magnetic resonance method at 60 °C per sec, with the use of samples of 0.33 and 0.5 ml. The protein concentration of the samples ranged from 4 to 12 mg per ml. Amounts of manganese added varied from 5.5 to 16.5 X 10⁻⁵ M.

At these manganese concentrations, the binding to Tris was negligible (22). The Mn²⁺ solutions were assayed by the permanganate method after oxidation with periodate (23). The temperature during the NMR measurements was 34° and was found to vary less than 1° during the experiment.

The longitudinal relaxation rates (1/T₁) were determined by the direct method (24, 25) with the use of either a time-averaging computer or Linograph direct print paper (Kodak) to record the signal. The difference between the NMR signal and its final asymptotic value was plotted against time on semilogarithmic paper. The relaxation time (T₁) was 0.434 times the time required for the ordinate to decrease by a factor of 10.

Because the formation of the arginase-manganese complex is temperature-dependent, enzyme samples were activated with Mn²⁺ by incubation for 10 min at 50° prior to NMR measurements.

**Evaluation of Enhancement, Number of Binding Sites, and Association Constants**—The enhancement $e^*$, which is defined as the ratio of proton relaxation rate of a manganese solution in the presence and absence of complexing agents, was calculated according to the following equation (25),

$$e^* = \frac{(1/T_{10})}{(1/T_{1(0)})}$$

where $T_1$ and $T_{1(0)}$ represent the observed longitudinal relaxation times of aqueous solutions with and without manganese, respectively, and the symbols with asterisks represent the same parameters in the presence of a complexing agent. Because the protein component of arginase is assumed to have negligible effect on the proton relaxation rate of water at the concentrations used, the relaxation rate obtained for $1/T_{1(0)}$ was used for the correction value ($1/T_{1(0)}$), which represents the proton relaxation rate of water, buffer, salt, and protein, in calculating the enhancement due to the tight Mn⁺ binding. This assumption is supported by published measurements on bovine serum albumin (7) and pyruvate kinase (8).

Since arginase has 2 tightly bound manganese atoms which do not noticeably participate in the equilibria, the enhancement ($e^*$) of the weakly bound manganese was determined by subtracting the effect due to the tightly bound manganese, i.e. for $1/T_{1(0)}$, the arginase-manganese value without added manganese was used, and the concentration in the denominator was that of the added manganese only.

Free and bound manganese and binding constants (K) were calculated by equations given in the literature (7, 25). These equations apply to identical and independent binding sites, which is not true for rat liver arginase; however, because $K_1$ is very large, its effect on the equilibria was assumed to be negligible. The enhancement for the binary complex ($e_b$) was primarily determined by linear extrapolation of the $e^*$ values. However, because this extrapolation is, in general, not linear, the results were also fitted to Equation 5C of Mildvan and Cohn (7).

**RESULTS**

**Removability of Manganese from Rat Liver Arginase**

Native rat liver arginase exists in a manganese-activated form. During the purification procedure, part of the bound manganese is dissociated from the enzyme. This was observed especially after purification steps such as alcohol fractionation and column chromatography. However, the catalytic activity could be increased considerably by subsequent manganese activation.

For the activation studies by kinetic and magnetic resonance measurements, it was necessary to remove as much manganese as possible from the enzyme complex, without causing irreversible denaturation or permanent loss of enzymatic activity. This was achieved by the following three procedures.

**Dialysis**—Dialysis of rat liver arginase against 0.01 M Tris-HCl buffer (pH 7.5) at 4° with daily changes of the dialysis buffer, showed a constant pattern in repeated experiments. Within about 30 hours, the catalytic activity dropped to approximately half of its original value. On continued dialysis, the activity level did not change noticeably for the next several days. After manganese activation, the original activity could be regained in all samples.

To obtain qualitative information about the binding of manganese the following experiment was performed. A previously dialyzed arginase solution (55% residual activity) was activated at pH 7.5 in 0.05 M MnCl₂ solution (containing traces of ⁵⁴Mn) by incubation in a 50° water bath for 10 min. Samples of this enzyme preparation were dialyzed for 1 to 7 days as described above. The remaining radioactivity, as well as the catalytic ac-
DAYS DIALYSIS

Fig. 1. Dialysis ofabenylabeled arginase from rat liver. Curve 1, residual activity of dialyzed samples; Curve 2, radioactive counts of dialyzed samples; Curve 3, activity after reactivations in 0.05 M MnCl₂ solution. The graph shows that after 2 days of dialysis, a plateau region is reached, where activity, as well as radioactive counts of the samples, remained fairly constant during several days of continued dialysis.

Activity with and without manganese activation of each of these samples, was determined. The results of these experiments are shown in Fig. 1. After dialyzing out the excess of Mn²⁺, the radioactive counts remained constant for a number of days (Curve 2). It should be mentioned that this result is supposed to be considered only in a qualitative sense with respect to manganese binding. The catalytic activity (Curve 1) dropped rapidly within 48 hours of dialysis to about 54% and decreased only slightly (~8%) during the residual dialysis time. These data strongly suggest that the remaining biological activity of arginase depends on enzyme-bound manganese. Full activity was recovered in all samples after manganese activation (Curve 3).

Further extensive dialysis for up to 3 weeks yielded an enzyme with approximately 30% of its original activity. However, part of the enzyme in the dialysis bag was precipitated, and it was not possible to regain catalytic activity of the precipitated enzyme after activation with Mn²⁺. In repeated dialysis experiments, it became apparent that with increasing dialysis time the enzyme permanently lost its catalytic activity and was irreversibly denatured.

Chelating Agents—Arginase samples were incubated with chelating agents in order to bind the manganese and remove it from the protein. Incubation of the enzyme in 10⁻³ M to 5 × 10⁻⁴ M EDTA solutions gradually decreased the catalytic activity. In a similar experiment, the enzyme decreased to nearly 50% of its original value after 2 days dialysis against a 10⁻² M EDTA solution (pH 7.2). Full activity was regained in all samples after metal ion activation.

Experiments with the chelating compounds diethylenetriamine pentaacetic acid and 2-hydroxyethylidenediacectic acid yielded the same results. The addition of 5 × 10⁻³ M to 5 × 10⁻⁴ M complexing agents caused a gradual reduction of the enzymatic activity of the fully activated enzyme preparation. However, even at diethylenetriamine pentaacetic acid and 2-hydroxyethylidenediacectic acid concentrations of 5 × 10⁻³ M, not more than 50% of the catalytic activity was lost due to chelation of manganese.

A similar result was obtained by column chromatography with Chelex 100, a chelating resin containing iminodiacetate functional groups attached to a styrene divinylbenzene lattice (26). The column (1 x 12 cm) was used in the sodium form, equilibrated and eluted with 0.05 M Tris-HCl buffer. A 0.2% solution of arginase (3 ml) was passed through the resin at a flow rate of 15 ml per hour. In two repeated experiments, carried out at pH 7.5, at which the imino and carboxyl groups are ionized, enzyme preparations with only about 50% of the original specific activity were obtained.

Electrofocusing—In connection with the previous experiments, it was interesting to observe that after electrofocusing for 64 hours in pH 3 to 10 and pH 7 to 10 gradients, the rat liver enzyme showed an activity that could be increased 2-fold on manganese activation (Fig. 2). This means again that during electrofocusing the weakly bound manganese was dissociated from the enzyme, which resulted in a loss of about 50% of the biological activity.

Activation at Increasing Manganese Concentrations

In order to find maximal activation conditions, previously dialyzed arginase samples were activated at various Mn²⁺ concentrations (0.005 to 0.1 M). The increase in catalytic activity versus manganese concentration is plotted in Fig. 3. Under the applied conditions (see legend to Fig. 3), full activation of the enzyme by Mn²⁺ was obtained at a concentration of ~0.04 M.

Fig. 2. Electrofocusing of rat liver arginase in a pH 3 to 10 gradient. The experiment was carried out as described under "Materials and Methods." Enzyme activity was determined with isonirosopropiophenone. Δ---Δ, arginase activity after electrofocusing; ○---○, arginase activity after reactivation in 0.05 M MnCl₂ solution at pH 7.5; ---, pH gradient.
Fig. 3. Activation of a dialyzed preparation of rat liver arginase at various manganese concentrations. The activation was carried out by incubation in a water bath at 50°C for 10 min. The incubation mixtures contained 0.05 M Tris-HCl buffer, pH 7.5. After incubation, the enzyme samples were chilled in ice and activity tests were performed at 30°C. Under the conditions described, full activation is obtained at a manganese concentration of >0.04 M.

Temperature and pH Dependence of Manganese Activation

Under conditions described for the above experiments (see legend to Fig. 3), previously dialyzed enzyme solutions with a residual catalytic activity of about 50% were activated in a 0.05 M MnCl₂ solution, pH 7.5, at 30°C, 40°C, and 55°C (Fig. 4). The experiment at 55°C shows that rat liver arginase is quite stable against heat denaturation, especially in the presence of a high concentration of manganese. At higher temperatures, a gradual irreversible decrease of the catalytic activity could be observed.

Manganese activation of the same samples at 40°C for 1 hour at various pH values (pH 6.0 to 10.5, 0.05 M Tris-HCl buffer) gave a wide plateau of maximum activity, which shows that the activation step is in the 7.0 to 9.0 pH range, nearly independent of pH.

Nuclear Magnetic Resonance Measurements

Determination by Nuclear Magnetic Resonance and Electron Spin Resonance of Enhancement (εₚ), Binding Constant (Kₐ), and Number of Tightly Bound Manganese—As the experiments with chelating agents, dialysis, and electrophoresis demonstrated, it was not possible to remove quantitatively the bound manganese from the enzyme without destroying irreversibly the activity and structure of the protein. Consequently, it was of interest to determine by NMR experiments how many Mn²⁺ are tightly bound by an arginase preparation which had lost approximately 50% of its original activity due to dissociation of manganese. Therefore, dialyzed enzyme samples (51% residual catalytic activity) were incubated in 1.6 M HCl as described under “Materials and Methods.” The incubation in the acid medium resulted in immediate irreversible denaturation and precipitated the protein. By continued shaking, the manganese was dissolved as Mn²⁺ into the supernatant solution. The samples were centrifuged and the precipitates washed with small amounts of HCl of the same concentration. The amount of free manganese in the HCl solution was then determined quantitatively by ESR together with several standards. Three determinations of arginase samples gave an average value of 2.00 ± 0.21 moles of tightly bound manganese per mole of enzyme.

For the measurement of the proton relaxation rates of the same enzyme preparation, no additional manganese was added. A rather strong increase of the proton relaxation rates (1/T₁*) compared to the effect caused by equal amounts of free manganese could be observed. In order to determine the enhancement εₚ, defined as the ratio of proton relaxation rate of manganese solution in the presence and absence of a complexing agent, the amount of manganese present in the enzyme samples was determined quantitatively by ESR, as described before. The value (1/T₁) for the corresponding amount of free manganese was then obtained from a standard curve. For εₚ, a value of 8.0 was obtained. The values for εₚ were found to change very little with increasing enzyme concentration. Therefore, the lower limit of the binding constant (Kₐ) was estimated and found to be 3 × 10⁷ M⁻¹.

Titration of Arginase with Manganese

Determination of Enhancement (εₚ) of Weakly Bound Manganese Ions, Association Constant (Kₐ), and Number of Binding Sites (n)—Arginase samples (4 × 10⁻⁵ M) were titrated with increasing amounts of manganese (5.5 to 16.5 × 10⁻⁵ M). A dialyzed enzyme preparation with 50% residual catalytic activity was used for these titrations. Prior to NMR measurements, the samples were activated at 50°C for 10 min. As determined by ESR, the arginase samples already had close to 8 × 10⁻² M manganese bound. The enhancement factors (εₚ) were calculated as described under “Materials and Methods.” In Fig. 5,
Fig. 5. Titration of a dialyzed arginase sample (residual activity 51%) with increasing concentrations of manganese (5.5 to 16.5 \times 10^{-5} \text{ M}). Each sample (0.33 ml) contained 4 \times 10^{-5} \text{ M arginase and 0.01 M Tris-HCl buffer, pH 7.5}. The graph shows the reciprocal of the observed enhancement due to the weakly bound manganese versus the concentration of manganese added. In the calculation of the enhancement factors, the effect caused by the tightly bound manganese was subtracted as described under "Materials and Methods." NMR measurements were carried out at 34° after activating the samples at 50° for 10 min.

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Fig. 6. Hughes and Klotz type of plot of the ratio of arginase to weakly bound manganese versus the reciprocal of the free manganese concentration.

A plot of the reciprocal enhancement (1/\epsilon^*) versus increasing manganese concentration is given.

The number of binding sites on the protein was determined graphically by a double reciprocal plot according to the method of Hughes and Klotz (27). The amount of free manganese was calculated from the enhancement data by means of the value of \epsilon_0 = 6. The MnO value was obtained by subtracting Mn from the total amount of manganese. From the intercept on the ordinate, two sites which bind Mn weakly were determined (Fig. 6). Because the extrapolation of \epsilon_0 may not be linear, the full equations given in Reference 7 were applied (assuming n = 2) for each point in Fig. 5. It was found that values of \epsilon_0 = 0 and \kappa_2 = 2 \pm 1 \times 10^4 \text{ M}^{-1} fit Equation 5C (7).

Fig. 7. Titration of Mn\(^{2+}\) (5.5 \times 10^{-5} \text{ M}) with dialyzed arginase preparation (residual activity 51%). Double reciprocal plot of the observed enhancement versus total arginase concentration. The samples (0.5 ml) contained 0.01 M Tris-HCl buffer, pH 7.5. The addition of 300 mM KCl did not cause a change in the observed enhancement. The activation of the sample, as well as the calculation of the enhancement factors, was carried out as described in the legend to Fig. 5.

**Titration of Manganese with Arginase**

Determination of Enhancement \epsilon_0—Manganese chloride (5.5 \times 10^{-5} \text{ M}) was titrated with variable amounts of arginase (4 \times 10^{-5} to 10^{-4} \text{ M}) in the presence of 0.01 M Tris-HCl buffer at pH 7.5.

The same enzyme preparation was used as for the previous experiment. Each sample was activated. In calculating the enhancement factors (\epsilon^*) of the weakly bound manganese, the effect caused by the tightly bound manganese was subtracted as described before. In Fig. 7, the reciprocal enhancement (1/\epsilon^*) obtained for each experiment is plotted against the reciprocal manganese concentration. Extrapolation to infinite manganese concentration, as well as application of the full equations (7), yielded an \epsilon_0 of 8.0. Assuming a binding constant of \kappa_2 = 2 \times 10^4 \text{ M}^{-1} and n = 2, the calculated theoretical enhancement values fit the experimental points well.

**Effect of Arginine and Ornithine on Enhancement**

Determination of \epsilon_1—The addition of 12 to 120 mM arginine or ornithine to the manganese-activated enzyme yielded slightly reduced values for \epsilon^*. Assuming the formation of a ternary complex E-manganese-arginine and E-manganese-ornithine, the enhancement (\epsilon_1) was determined according to Procedure I of Mildvan and Cohn (28). From a plot of 1/\epsilon^* versus the reciprocal arginine or ornithine concentration, values for \epsilon_1 for three different enzyme concentrations were obtained. When 1/\epsilon_0^* was plotted against the reciprocal arginine concentration, an enhancement of \epsilon_1 = 1.7 for the E-manganese-arginine complex and \epsilon_1 = 7.5 for the E-manganese-ornithine complex was extrapolated. These values, obtained by linear extrapolation, represent the upper limits for \epsilon_1.

**DISCUSSION**

The catalytic activity of rat liver arginase depends, to a large degree, on the presence of Mn\(^{2+}\) ions. During certain purification steps, e.g. column chromatography, alcohol fractionation, and dialysis, part of the metal ion is lost, and the specific activ-
ity drops by as much as 50%. A loss of enzymatic activity is also observed after extreme dilution of the enzyme (≈0.01 μg of protein per ml).

Attempts to remove all the manganese reversibly from this enzyme have been unsuccessful. There are many examples of enzymes which require, for catalytic activity, the addition of a divalent metal ion such as Mn²⁺, e.g. kinases, enolases, and some dehydrogenases, but in these cases the manganese is readily dissociable. There are very few reports of a highly purified enzyme which contains non-dissociable manganese (29, 30).

A correlation between the removal of Mn²⁺ from the arginase complex and the loss in catalytic activity could be demonstrated by dialysis experiments using ⁴⁴Mn isotope as a tracer. Part of the manganese of an enzyme sample labeled with ⁴⁴Mn was easily removed by dialysis; however, as can be seen from the data (Fig. 1), a plateau region is reached after about 48 hours of dialysis and approximately 50% of the catalytic activity of the enzyme remains. At this point, the original catalytic activity can be completely regained after activation with Mn²⁺. The ⁴⁴Mn content of the sample stays fairly constant on continued dialysis for several days; in addition, the rate of inactivation of the enzyme is small. Similar results with respect to the loss of activity due to manganese dissociation and recovery of activity by metal activation were obtained by other methods, such as electrophoresis (Fig. 2), chelating agents, and chromatography on a Chelex 100 chelating resin column.

After dialysis for 1 week or longer, the enzyme starts to precipitate in the dialysis bag, and the inactivation of the arginase is no longer reversible; the original activity of the precipitated enzyme cannot be regained fully by activation with Mn²⁺ ions. It appears that the loss of the residual Mn²⁺ during prolonged dialysis causes an irreversible conformational change in the structure of the protein molecule, resulting in instability and denaturation of the enzyme.

ESR measurements after denaturation of arginase by 1.5 N HCl showed that a dialyzed enzyme sample with remaining activity of 51% contained approximately 2 moles of manganese per 118,000 daltons, suggesting that the enzyme then exists as an E-M₂ complex. From measurements of the proton relaxation rates, an enhancement (εₚ) of 8.0 was determined, and the lower value of the binding constant (K₁) for the E-M₂ complex was found to be 3 × 10⁹ M⁻¹.

In titration experiments of dialyzed arginase (E-M₂) with Mn²⁺ solutions, it was shown by NMR data that 2 additional manganese atoms can be bound by the enzyme. The addition of 300 mM KCl did not cause a change in the observed enhancement, indicating a specific binding of manganese. The binding constant (K₂) was determined to be 2 ± 1 × 10⁹ M⁻¹. Consequently, the fully biologically active enzyme exists in an E-M₄ form. The binding constants for the tightly and weakly bound manganese, however, differ by a factor of more than 10³. However, despite the different binding constants observed for the binding of the Mn²⁺ ions to arginase, we believe that they do not represent the intrinsic binding constants. Rather, we think that the difference between K₁ and K₂ is due to a change in the structure of the enzyme caused by the loss of 2 Mn²⁺ ions per protein molecule. This assumption is supported by the results obtained from the dialysis experiment using ⁴⁴Mn. After activation of a dialyzed enzyme sample at 50°C, radioactive manganese was incorporated into the enzyme and part of it remained firmly bound during subsequent dialysis. This exchange of manganese suggests that in the fully active enzyme weak and tight manganese binding sites are probably not fixed within the enzyme molecule, but that after dissociation of 2 manganese ions, the enzyme undergoes a conformational change which causes a tighter binding of the residual 2 manganese ions.

Another possible explanation of these data is that at 50°C the tightly bound manganese is loosened and exchanged by the manganese isotope. However, the fact that the catalytic activity of arginase is rather stable at 50°C, even in the absence of additional manganese, favors the first explanation.

The addition of the substrate arginine or the competitive inhibitor ornithine to the arginase-manganese complex did not increase the enhancement. The fact that εₚ, the enhancement of the ternary complex, is not larger than εₚ indicates that primarily a binary complex between the enzyme and the metal ion is formed, a suggestion which has previously been made by Mohamed and Greenberg from activity studies with horse liver arginase (31). Therefore, arginase belongs to type II of the metal-catalyzed enzyme reactions described by Cohn (32).

The activation of the E-M₂ ≈ E-M₄ complex proved to be a very slow reaction compared to many other manganese-dependent enzymes, e.g. pyruvate kinase (8) and enolase (33), in which, immediately after the addition of the manganese solution, the biological activity reaches its full value and no further heating is required. In the case of arginase (Fig. 4), the manganese activation is temperature-dependent. At room temperature, a previously dialyzed enzyme sample required about 8 to 10 hours incubation with manganese to obtain full activity. By raising the temperature to 65°C, the reactivation of the enzyme, which is the formation of the E-M₄ complex, was completed within 5 min.

Between pH 7.0 and 9.0, the reactivation step was shown to be almost independent of pH. In case of the binding of manganese by two ligands on the surface of the protein molecule, one would expect a fast reaction and possibly a pH dependence. The results of these activation studies, therefore, support the idea that a change in the structure of the enzyme, which is comparatively slow at room temperature, is the rate-limiting step in the manganese activation of rat liver arginase. As has been concluded from activation studies with divalent metal ions (6) on bovine liver arginase, the particular site of metal ion binding is evidently not readily accessible. Together with the comparatively high value observed for the enhancement (εₚ) of the arginase-manganese complex, the results suggest that the manganese may be bound in a pocket of the protein molecule.

In conclusion, rat liver arginase, which binds 4 moles of manganese per mole of enzyme is composed of 4 subunits (15) and has four binding sites for the competitive inhibitor ornithine (34). Consequently, it can be assumed that arginase has four active sites with one Mn²⁺ bound per site.
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