The Mechanism of Aconitase Action

II. MAGNETIC RESONANCE STUDIES OF THE COMPLEXES OF ENZYME, MANGANESE(II), IRON(II), AND SUBSTRATES*

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SUMMARY

When a number of metal ions were tested as activators over a 100-fold range of metal ion concentration (0.01 to 1.0 mM), aconitase was specifically activated by Fe(II). Manganese(II) does not activate aconitase but binds to the enzyme, as shown indirectly by inhibition of the enzyme activation by Fe(II) and directly by electron paramagnetic resonance. A titration of the enzyme with Mn(II) may be fit most simply by assuming two "tight" binding sites for Mn(II) with a dissociation constant, $K_{diss}$ of $47 \pm 3$ $\mu$M and 5 to 7 "weak" binding sites ($K_{diss} = 620 \pm 80$ $\mu$M). Interaction among these sites is indicated by the effect of bound Mn(II) on the longitudinal relaxation rate ($1/T_{1P}$) of water protons. Iron(II) competes with Mn(II) for the tight binding sites, yielding a dissociation constant for aconitase-Fe(II) of $16 \pm 6$ $\mu$M. The binary aconitase-Fe(II) complex and the ternary aconitase-Fe(II)-citrate complex are paramagnetic, as determined by $1/T_{1P}$ of water protons, but less so than unbound Fe(II), as determined by magnetic susceptibility. In the catalytically inactive Mn(aconitase) complex, a 3-fold enhancement of the effect of Mn on $1/T_{1P}$ of water protons is observed. Citrate, isocitrate, and cis-aconitate halve this enhancement, suggesting the formation of ternary enzyme-metal-substrate complex at the catalytic center intimately involved with the enzymatic reactions. The choice between these two coordination schemes may be made by magnetic resonance studies with purified enzymes.

Many attempts have been made to determine whether the reactions of Equation 1 are catalyzed by a single or dual catalytic center(s). The bulk of the evidence indicates a single catalytic center to be responsible for all of the interconversions, although this conclusion has recently been questioned (7). Another purpose of the binding studies (in addition to investigating the coordination scheme by binding studies with Mn(II) and Fe(II)).

EXPERIMENTAL PROCEDURE

Materials

Monopotassium three-o-($\pm$)-isocitrate acid and cis-aconitic acid, the reverse reactions, and the interconversion of citric and isocitric acids:

\[
\begin{align*}
\text{cis-Aconitate} & \quad \text{Citrate} \\
\text{Isocitrate} & \quad \text{cis-Aconitate}
\end{align*}
\]

Dickman and Cloutier (1) determined that aconitase requires ferrous ion and a reducing agent for maximum activity. The role of this metal ion in the catalytic activity of aconitase, however, is unknown. Of the three possible coordination schemes of ternary complexes (2, 3), substrate bridge ($E-S-M$), metal bridge ($E-M-S$), and enzyme bridge ($M-E-S$), a substrate bridge complex may be excluded by the observation that incubation of the enzyme with Fe(II) is required for activation (4, 5). However, it remains unclear whether the divalent cation is involved in a metal-enzyme-substrate complex in a structural role distant from the catalytic center, or whether it is involved in an enzyme-metal-substrate complex at the catalytic center intimately involved with the enzymatic reactions. The choice between these two coordination schemes may be made by magnetic resonance studies with purified enzymes (2). Pig heart aconitase has recently been purified (6), permitting the present study of its coordination scheme by binding studies with Mn(II) and Fe(II).

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Many attempts have been made to determine whether the reactions of Equation 1 are catalyzed by a single or dual catalytic center(s). The bulk of the evidence indicates a single catalytic center to be responsible for all of the interconversions, although this conclusion has recently been questioned (7). Another purpose of the binding studies (in addition to investigating the coordination scheme) in this work is to determine whether these reactions are catalyzed at a single catalytic center or at dual active sites.

EXPERIMENTAL PROCEDURE

Materials

Monopotassium three-o-($\pm$)-isocitrate acid and cis-aconitic acid were purchased from Sigma and citric acid from Calbiochem. RuCl₃ and RhCl₃ were products of Alfa Inorganics, Beverly, Massachusetts, and Gd(NO₃)₃ was purchased from Chemicals Procurement Laboratories, College Point, New York. All other chemicals were the purest grade commercial products available. Deionized water was used throughout, and all buffers and reagents were checked for paramagnetic contaminants by meas-
uring the longitudinal relaxation rate, $1/T_1$, of the protons of water by pulsed NMR at 24.3 MHz.

**Methods**

**Enzyme Assays**—Aconitase was purified to homogeneity as previously described (6). The specific activity of the enzyme was 9 μmoles of isocitrate produced from citrate per min per mg of enzyme. For binding studies, the molecular weight of the enzyme was assumed to be 89,000 (6). For kinetic studies, with citrate and cis-aconitate as substrates, the enzyme was assayed by following the production of NADH at 340 nm on a Gilford 240 recording spectrophotometer (8). An assay based on the ultraviolet absorption of cis-aconitate at 240 nm was used with isocitrate as the substrate, as previously described (9).

When various metal ions were examined for activation of aconitase, the enzyme (2.5 mg per ml) was added to a solution containing 0.01 to 1.0 mM of the metal salt; 40 mM sodium N-2-hydroxyethylpiperazine-N-2-ethane sulfonate, pH 7.5; and 10 mM cysteine, pH 7.5 (when present), in a final volume of 0.1 ml (6). This solution was incubated for 30 min, and a 20-μl aliquot was removed and assayed for activity by means of the 340 nm assay described above with citrate as the substrate (8).

**Magnetic Resonance Techniques**—The concentration of free Mn(II) was determined by the method of Cohn and Townsend (10) from the amplitude of the EPR spectrum of Mn(II) obtained on a Varian E-4 EPR spectrometer. The sample (30 to 50 μl) was contained in a quartz capillary tube maintained at 25° for the measurement.

The longitudinal relaxation rate, $1/T_1$, of the protons of water was measured in a Nuclear Magnetic Resonance Specialties PS60-W pulsed NMR spectrometer operating at 24.3 MHz, as previously described by Mildvan and Cohn (11). The observed enhancement, $e^*$, of the paramagnetic contribution to the longitudinal relaxation rate and the enhancement, $e_b$, due to complexed Mn(II) were calculated as described by Mildvan and Cohn (11).

The magnetic susceptibility was determined by an NMR method with the use of the Varian 220 MHz NMR spectrometer (12) with 0.4-ml samples containing 0.96 μmoles aconitase; 0.68, and 0.90 mM Fe(NH$_4$)$_2$SO$_4$; 0.4, 4.8, and 9.1 mM cysteine; 11 mM tetramethylammonium chloride; and 0.1 mM Tris-DCI, pH 7.5 in 99% D$_2$O. The chemical shift of the internal tetramethylammonium resonance, which was measured with respect to external tetramethylsilane, is directly proportional to the bulk magnetic susceptibility of the sample. Reference solutions of known magnetic susceptibility, containing MnCl$_2$ in the presence and absence of cysteine and bovine plasma albumin, were used for calibrations. The molar paramagnetic susceptibility of MnCl$_2$ solutions was unchanged by cysteine or albumin and was assumed to be 14,600 × 10$^{-6}$ c.g.s. units (13).

**Results**

**Metal Specificity of Aconitase**—Homogeneous aconitase requires Fe(II) and cysteine for activity (6), as found previously by Dickman and Cloutier (1) and Morrison (5) with partially purified enzyme. Other cations were tested at a single concentration (0 mM) as activators of aconitase (1), but none could replace Fe(II). It is possible that at this high concentration the other metals might inhibit the enzyme, whereas at lower concentrations activation might occur. Inactivation by high concentrations of divalent cations has been discussed in a review by Eichorn (14).

The divalent cations listed in Table I were, therefore, tested over a 100-fold concentration range (0.01 to 1.0 mM) in the presence and absence of cysteine to determine whether they can activate aconitase. The results in Table I show that none of the metals produced an activation greater than 1%, and it is concluded that none of the metals tested can replace Fe(II) in the activation of aconitase.

Because of its value as a paramagnetic probe (2), the binding of Mn(II) was initially studied by its ability to inhibit the activation of aconitase by Fe(II). When MnCl$_2$ was present in the incubation mixture at concentrations comparable with Fe(NH$_4$)$_2$SO$_4$ (0.5 mM), the time required for half-maximal activation of aconitase doubled. A similar effect was observed with a 50-fold excess of Mn(II) over 0.1 mM Fe(II), and a tripling of the half-time for activation was observed with a 100-fold excess of Mn(II) over 0.1 mM Fe(II). The final extent of activation, however, was reduced by only 10 to 20% in these experiments. Thus, Mn(II) interacts with aconitase and inhibits its activation by Fe(II). However, since the activation of aconitase by Fe(II) is a complicated process involving two Fe(II) binding steps and possible conformational changes (6), no further analysis of these kinetic data was attempted.

**Binding of Manganese(II) to Aconitase**—A direct study of the binding of Mn(II) to aconitase was made by EPR and PRR. In Fig. 1A, the binding data from the EPR study are presented in the form of a Scatchard plot. The curved line suggests that more than one set of Mn(II) binding sites exist on aconitase, and the data were analyzed by the method of Scatchard (15) with the use of Equation 2 for two thermodynamically noninteracting sets of Mn(II) binding sites.

\[
\frac{(\text{Mn})_{\text{II}}}{(\text{E})_{\text{f}}} = \frac{n_1}{K_1} + \frac{n_2 + n_0}{K_2} + \frac{n_1 + n_0}{K_1 K_2}
\]

The subscripts $f$ and $b$ refer to free and bound Mn(II), respectively; $(\text{Mn})_{\text{II}}$ and $(\text{Mn})_{\text{f}}$ refer to Mn(II) bound at sites 1 and 2, respectively; $(\text{E})_{\text{f}}$ is the total enzyme concentration, $n_1$ is the number of Mn(II) sites with a dissociation constant $K_1$, and $n_2$ is the number of sites with a dissociation constant $K_2$. The curved line in Fig. 1A can be reproduced by the combination of two lines, each representing a set of equivalent Mn(II) binding sites. One line gives a set of 2.2 ± 0.2 tight Mn(II) binding sites with a dissociation constant $K_1 = 47 ± 3 \mu M$, whereas the other line gives $6.6 ± 1$ weak Mn(II) sites with $K_2 = 620 ± 80 \mu M$. The number of weak sites is less precise since the curved line asymptotically approaches the abscissa, preventing an accurate determination of the intercept.

The enhancement of the PRR of water due to bound manganese ($e_b$) decreases with an increase in occupancy of the manganese binding sites from a value of $e_b = 3.0 ± 0.2$ when extrapolated to $(\text{Mn})_{\text{II}}/(\text{E})_{\text{f}} = 0$ to a value of $1.6 ± 0.1$ when more than two sites are occupied (Fig. 1B). The former value ($3.0 ± 0.2$) represents $e_{01}$, the enhancement of the tight binding sites when the weak sites are not occupied. A separate analysis of the data by simultaneous equations yields a value of $e_{01} = 3.4 ± 0.6$. If the value of $e_{01}$ is assumed to be constant over the entire range of

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1 The abbreviations used are: NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; PRR, proton relaxation rate.
TABLE I
Effect of various metals on activation of aconitase

| Metal salt    | Cysteine | Percentage of activity |
|---------------|----------|------------------------|
|               | 1.0 mm   | 0.1 mm                 | 0.01 mm |
| Fe (NH₄SO₄)₂  | +        | 100                    | 90      | 80      |
| MnCl₂         | -        | -                      | -       | 0       |
| CoCl₂         | +        | 16                     | 10      | 0       |
| Ni (NO₃)₆     | +        | -                      | -       | 0       |
| Cu (CH₃CO₂)₂  | +        | -                      | -       | 0       |
| ZnCl₂         | +        | 1                      | 1       | 0       |
| MgCl₂         | +        | 0                      | 0       | 0       |
| CdCl₂         | +        | 0                      | 0       | 0       |
| CaCl₂         | +        | -                      | -       | 0       |
| RuCl₃         | +        | -                      | -       | 0       |
| RhCl₃         | +        | -                      | -       | 0       |
| Gd (NO₃)₃     | +        | 0                      | 0       | 0       |

* The assay method of Rose and O'Connell (8) was used. Metal was incubated with aconitase for 30 min at 25° as described under "Methods."

** A final concentration of 10 μM cysteine was present in the incubation mixture where noted by a plus sign.

All activities are normalized to aconitase activated in 1.0 mM Fe(II)-10 mM cysteine for 30 min at 25°. A zero value listed in this table represents the lower limit of detection of the assay used (<0.1%).

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* Final concentrations of Ni(NO₃)₆ were 3.2, 0.32, and 0.032 mM.

* A precipitate formed in the incubation mixture; the activity was not determined.

* Final concentrations of CdCl₂ were 1.1, 0.11, and 0.011 mM.

* When cysteine is added to Ru(III), it is reduced to Ru(I).

the titration (3.0 ± 0.2) (Fig. 1A), the enhancement of the weak binding sites, ε₂, calculated from Equation 3,

\[ ε₂ = \frac{(Mn)₂}{(Mn)₃} + \frac{(Mn)₃}{(Mn)₄} \]

drifts progressively downward from a limiting value of 1.8 ± 0.3 at \((Mn)_3/(E)_4 = 0\), reaching negative values when 0.9 tight binding site and 0.1 weak binding site are occupied (Table II). Since negative values of enhancement are physically impossible, the analysis indicates that \(ε₂\) is not constant.

Competition Between Iron(II) and Manganese(II) for Metal Sites of Aconitase—To determine whether the Mn(II) was bound at the same site(s) as Fe(II) on aconitase, solutions of Mn(II) and enzyme were titrated with Fe(II), and the free Mn(II) was measured by EPR. As shown in Table III, the fraction of Mn(II) which is free increases as the concentration of Fe(II) increases. The dissociation constant for the Fe(II)-enzyme complex can be calculated from the EPR data by the method of Hastings et al. (16) in a manner similar to that used by Mildvan and Cohn (17) for magnesium-pyruvate kinase. The first calculation assumed competition between the two metals for the tight sites on the enzyme. The dissociation constant for the Fe(II)-enzyme complex, \(K_{Fe}^{Mn}\) calculated in this manner shows a slight upward drift as the concentration of Fe(II) is increased (Table III). Such behavior was observed in the interaction of Ca(II) with the magnesium-pyruvate kinase complex (17). Hence, in a separate analysis, a correction was made for the contribution of Fe(II) binding to the weak set of Mn(II) binding sites on aconitase. To make this correction, the simplifying assumption was
made that the ratio of Mn(II) bound to the tight and weak metal binding sites on aconitase remains the same in the presence of Fe(II), which is equivalent to the assumption that the relative affinity of Fe(II) for the tight and weak sets of Mn(II) binding sites is the same (≈13:1) as the relative affinity of Mn(II) for these sites (2, 11). The dissociation constant for the Fe(II)-enzyme complex for the tight metal binding sites, when calculated in this manner (Table III, last column), is fairly constant for all Fe(II) to aconitase ratios. The average value for the KdFe is a factor of 2 lower when the correction for the contribution of the in this manner (Table III, last column), is fairly constant for all Fe(II) to aconitase ratios. The average value for the KdFe is a factor of 2 lower when the correction for the contribution of the

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**Water Proton Relaxation Rate Studies of Ternary Complexes of Aconitase, Manganese, and Substrates**—The presence of Mn(II) at the Fe(II) binding sites of aconitase and the absence of enzymatic activity permit a study of the interaction of each substrate with the tight manganese-aconitase complex. A solution containing 0.141 mM Mn(II) and 1.88 mM aconitase (Mn(II) / (E)_t = 0.1) was titrated with an identical solution which also contained 1.8 mM citrate, and the enhancement of the PRR of water was measured (Fig. 2). The observed enhancement, e*, decreased from an initial value of 2.6 to an end point of 1.4. No significant change occurred in the fraction of manganese which was free (5%), as determined by EPR. Hence, the results are consistent with the formation of a ternary complex of aconitase, manganese, and citrate.

The alternative possibility, that citrate merely removes Mn(II) from aconitase to form a binary manganese-citrate complex, is excluded by three observations. (a) The dissociation constant of manganese-citrate (K1 = 300 ± 10 μM) (18) is 6.4-fold greater than the K_diss of the manganese-aconitase complex. Hence, if no ternary complex had formed, citrate would produce a halving of the e* value by removing manganese from the enzyme when the citrate concentration is 6 times that of aconitase. End points were reached at concentrations of citrate (and of all other substrates) <3 times the aconitase concentration. (b) The value of e* at the end point (1.4 ± 0.1) is significantly greater than the enhancement of the binary manganese-citrate complex (e* = 1.0 ± 0.1), which was determined separately by PRR. (c) The value of e* at the end point (1.4 ± 0.1) was independent of aconitase concentration over a 3.5-fold range from 0.40 to 1.38 mM aconitase site. Hence, the titration data could be analyzed graphically by Procedure III, as previously described (19), to yield a dissociation constant which is the average of seven titrations.

$$K_1 = \frac{\text{(aconitase-manganese)(citrate)}}{\text{(aconitase-citrate)}} = 0.39 \pm 0.10 \text{mM} \quad (4)$$

The enhancement of the ternary complex (e*) is obtained from the end point of the titration at high concentrations of aconitase to be 1.4 ± 0.1 (Table IV). Such simple behavior has been observed in titrations of the pyruvate kinase-manganese complex with phosphoenolpyruvate (19) and its analogues (20).

Similar titrations were performed with isocitrate and cis-aconitate, and ternary complexes were detected by the same three criteria. Thus, the dissociation constants of the binary manganese-isocitrate and manganese-cis-aconitate complexes are 400 μM and

### Table III

**Apparent dissociation constants of Fe(II)-aconitase**

| (Mn(II)/E)_t | KdFe | KdFe\(^a\) |
|--------------|------|------------|
| 0.38         | 2.8  | 0.125 0.200 0.049 | 0.015 | 0.011 |
| 0.18         | 2.60 | 0.695 0.125 0.000 | 0.021 | 0.018 |
| 0.14         | 2.60 | 0.875 0.125 0.006 | 0.021 | 0.018 |
| 0.13         | 2.70 | 0.980 0.125 0.100 | 0.021 | 0.021 |
| 0.11         | 3.10 | 0.290 0.125 0.146 | 0.025 | 0.024 |
| 0.09         | 2.92 | 0.500 0.125 0.107 | 0.035 | 0.024 |
| 0.32         | 2.10 | 0.370 0.090 0.039 | 0.039 | 0.017 |
| 1.13         | 0.620 | 0.758 0.23 0.497 | 0.062 | 0.010 |
| 0.77         | 1.244 | 0.685 0.19 0.538 | 0.062 | 0.011 |

Average: 0.035 ± 0.023, 0.016 ± 0.006

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*a* The conditions for this experiment are the same as listed in Fig. 1 with the use of the concentrations of Mn(II), (E)_t, and Fe(II) listed in this table. The solutions were allowed to stand 15 min before 1/T_1 and (Mn)_f were measured to permit the reaction with Fe(II) to proceed to completion (6). Dithiothreitol (2 mM) was added in each of the above experiments to keep the Fe(II) reduced at pH 7.5. Dithiothreitol had no effect on the amplitude of the Mn(II) EPR signal, indicating that it does not bind to Mn(II).

*b* Calculated by assuming competition with manganese only at the tight binding site.

*c* Calculated by assuming competition with manganese at both tight and weak sites, as described in the text.

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**Fig. 2. Titration of manganese-aconitase with citrate.** In a typical experiment, a 30-μl solution of aconitase, 1.20 mM active sites, and MnCl₂ (144 μM) in 0.05 M triethanolamine chloride, pH 7.5, was titrated with an identical solution but which also contained 3.5 μM potassium citrate. After nearly complete titration by this solution, small additions of concentrated potassium citrate were added to reach an end point and the relaxation time was corrected for the small dilution. The 1/T_1 of water protons was measured and e* was calculated as described in Reference 11. The temperature was 22°C.
However, 25% of the cis-aconitate was converted to products no correction to the dissociation constant for isocitrate was made. The magnitude of the dissociation constant (K_a) of cis-aconitate (0.53 mM) is still more than an order of magnitude greater than the Km of cis-aconitate. The 1/T1 of water protons was measured as described under "Methods." The temperature was 22°C.

Iron(II)-activated aconitase was used for these experiments, and the assays used for each substrate are described under "Methods." The temperature was 25°C. K_m values were determined at four to five concentrations of each substrate over a 20- to 50-fold range, including the K_m by double reciprocal plots.

3400 ìM, respectively (18), which are much greater than the K_m of manganese-aconitase. Similarly, the K_m values of isocitrate and cis-aconitate are significantly greater than 1.0 and independent of enzyme concentration. The dissociation constants (K_a) and enhancement values (ε_i) of the ternary complexes are summarized in Table IV, where it is seen that the ε_i values of each substrate are half the enhancement of the tight binary aconitate-manganese complex (ε_i = 3.0). Such de-enhancements are consistent with the formation of aconitate-manganese-substrate bridge complexes in which water ligands on the enzyme-bound manganese have been replaced by substrate ligands (2).

At equilibrium, with the Fe(II)-activated enzyme, the substrates of aconitase are present in these amounts: citrate (90%), isocitrate (6%), and cis-aconitate (4%) (21). The enzyme used for these titrations retained 0.1% of activity in the absence of Fe(II) and cysteine (6). During the course of the titrations with isocitrate, less than 5% of the isocitrate was lost. Hence, no correction to the dissociation constant for isocitrate was made. However, 25% of the cis-aconitate was converted to products (primarily citrate). Hence, the observed dissociation constant for cis-aconitate from the enzyme-manganese-cis-aconitate complex (1.25 mM) must be corrected to a value of 0.94 mM if no competition with citrate occurred, or to a value of 0.53 mM if citrate and cis-aconitate compete for the same manganese site (see under "Discussion"). The lowest corrected value for the K_i of cis-aconitate (0.53 mM) is still more than an order of magnitude greater than the K_m of cis-aconitate.

Water Proton Relaxation Rate Study of Aconitase-Iron(II)-Citrate Complex—Little or no enhancement of the effect of Fe(II) on the PRR of water in iron complexes is expected since the correlation time for the dipolar contribution to the relaxation rate is dominated by the electron spin relaxation time both for the aquo complex and on macromolecules (22). In the presence of Fe(II) and cysteine, aconitase de-enhances the effect of the cation on the PRR of water protons (Table V). The extent of the de-enhancement appears to be time-dependent, which could reflect a change in the structure of the Fe(II) binding sites consistent with the time-dependent activation of aconitase by Fe(II) (6). The molar relaxivity (1/T1) of Fe(II) is decreased by ~60% in the aconitate-Fe(II) complex. This decrease in relaxivity is in part due to the displacement of water ligands of Fe(II) by ligands from the protein, and also due to a 42 ± 5% decrease in the paramagnetic susceptibility of bound Fe(II), from a control value of 9050 × 10^-6 c.g.s. units for unbound Fe(II). The paramagnetic susceptibility was measured by NMR under conditions similar to those shown in Table V, Experiment B. The latter observation suggests the formation of some low spin Fe(II) on binding to aconitase. A small increase in relaxivity occurs on adding a saturating level of citrate, and the relaxation rate remains constant. The paramagnetic susceptibility does not change from that of the binary complex. The paramagnetic relaxivity of the binary aconitate-Fe(II) complex and the ternary aconitate-Fe(II)-substrate complex (90% citrate at equilibrium) indicates that Fe(II) remains ~60% paramagnetic (high spin) in the presence of enzyme, cysteine, and substrates.

Michaelis Constants of Substrates of Aconitase—The Michaelis constants (K_m) of the three substrates of Fe(II)-activated aconitase were determined for the homogeneous aconitase preparation and are given in Table IV. The present K_m values are 2 to 5 times lower than those reported earlier for pig heart aconitase (23) and may represent a failure of the earlier workers to measure the true initial velocities at low substrate concentrations, since much of the earlier work was done with fixed time assays and we observe the rates to decrease within the time intervals previously used. Table IV also compares the dissociation constants of the iron-activated enzyme with those of the aconitate-Mn(II)-substrate complexes (K_i) with the respective K_m values of the substrates with Fe(II)-activated aconitase. The K_i values for citrate and isocitrate are in good agreement with their K_m values, but the K_i value for cis-aconitate is two orders of magnitude greater than its K_m, suggesting a different mode of binding of cis-aconitate to the Fe(II) and Mn(II) complexes of the enzyme.

Competition Between Citrate and Isocitrate for Aconitase-Manganese Complex—To determine whether citrate and isocitrate bind at the same metal site of aconitase, the enzyme-manganese complex was titrated with isocitrate at two fixed concentrations of citrate. Citrate levels were chosen which produced a partial de-enhancement of the water relaxation rate, due to formation of some E-manganese-citrate complex, and this mixture was

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**TABLE IV**

Dissociation constants of E-manganese-S ternary complexes and K_m values for substrates

| Substrate       | ε_i | K_a | K_m |
|-----------------|-----|-----|-----|
| Citrate         | 1.4 | 0.39 ± 0.1 | 0.62 ± 0.10 |
| Isocitrate      | 1.5 | 0.57 ± 0.1 | 0.20 ± 0.06 |
| cis-Aconitate   | 1.6 | 0.53 ± 0.1 | 0.015 ± 0.005 |

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**TABLE V**

Effect of iron(II) and aconitase on proton relaxation rate of water

| Experiment | Fe(II) | Aconitase sites | Citrate | Cysteine | T1/T1, s^(-1) | s^(-1) |
|------------|--------|----------------|---------|----------|---------------|-------|
| A          | 0.19   | 0.15           | 9.4     | 1.9      | 674           | 0.4   |
| B          | 0.50   | 1.11           | 10.2    | 772      | 1110 (15')    | 0.6   |

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*p These values were corrected for the water relaxation due to the bound Fe(II) of aconitase (6).
titrated with isocitrate until no further decrease in enhancement was observed. The results in Table VI, Column 5, indicate that the concentration of isocitrate needed to half-saturate the $E$-manganese complex, $S^2$, increases in the presence of added citrate. This behavior agrees with that predicted for competition (Table VI, Column 4), but not with that predicted for independent binding of citrate and isocitrate (Table VI, Column 3). The observed $e_T$ value also agrees better with the value predicted for competition than with the values predicted for no competition. Moreover, the value of $K_{a_{iso}}$ (Table VI, Column 6), when calculated assuming competition between citrate and isocitrate for $E$-manganese (see under "Discussion"), is constant and agrees within experimental error with the value of $K_{a_{iso}}$ obtained by direct titration (Table IV).

### DISCUSSION

The data of Fig. 1A were fit by assuming two sets of thermodynamically noninteracting Mn(II) binding sites in accord with Equation 2. These same data may, of course, be fit equally well by more complicated treatments involving the assumption of $8 \pm 1$ thermodynamically interacting binding sites with negative cooperativity among them (24). The dissociation constants for all sites would be equal but would decrease progressively as the site occupancy increased. More complicated assumptions are not required by the data. However, site-site interaction between the two tight sites, which has no effect on the dissociation constant, is suggested from an analysis of the PRR data. When the value of $e_{11}$ was assumed constant and a value for $e_{12}$ calculated from Equation 3, negative values of $e_{12}$ were calculated which indicated that this analysis was wrong and that a change in $e_{11}$ occurs. Hence, the data in Fig. 1D suggest that site-site interaction occurs which lowers the enhancement, $e_{11}$, at the tight binding sites as the weak sites are occupied. Such site-site interaction, which is not required to fit the thermodynamic data (Fig. 1A), has previously been reported for the binding of a ligand to alcohol dehydrogenase (25) and male dehydrogenase (26).²

² The presence of structural interaction among metal binding sites which may not manifest itself in the binding constants represents no inconsistency in our analysis. Thus, if the binding of manganese at site $b$ were to cause an opening of the manganese binding site $a$ such that the rate constant for manganese binding at site $a$ ($k_{a1}$) and for dissociation of manganese from site $a$ ($k_{a2}$) were increased by the same factor, the thermodynamic dissociation constant for manganese at site $a$ ($K_{a_{iso}} = k_{a1}/k_{a2}$) would be unaffected. However, the structural environment of bound manganese at site $a$ would be altered, resulting in a change (decrease) in $e_{11}$.

### Table VI

Dissociation constants for $E$-manganese-isocitrate in presence and absence of citrate

| Citrate Parameter and units | No competition | Competition | Observed | $K_{a_{iso}}$ |
|-----------------------------|----------------|-------------|----------|---------------|
| $K_d$ (mM) $e_T$             |                | 0.64        | 0.57     |               |
| 0.625 $K_a$ (mM) $e_T$       | 0.00           | 1.55        | 1.25     | 0.40          |
| 1.25 $K_b$ (mM) $e_T$        | 0.60           | 2.40        | 2.51     | 0.59          |

² A solution of aconitase (1.38 mm active sites), MnCl₂ (144 mM), and the indicated concentration of citrate in 0.05 M triethanolamine chloride, pH 7.5, was titrated with an identical solution, which also contained isocitrate over a range of concentrations of 1 to 4 mM. The $1/T_1$ of water protons was measured as described under "Methods." The temperature was 22°C.

³ The presence of structural interaction among metal binding sites which may not manifest itself in the binding constants represents no inconsistency in our analysis. Thus, if the binding of manganese at site $b$ were to cause an opening of the manganese binding site $a$ such that the rate constant for manganese binding at site $a$ ($k_{a1}$) and for dissociation of manganese from site $a$ ($k_{a2}$) were increased by the same factor, the thermodynamic dissociation constant for manganese at site $a$ ($K_{a_{iso}} = k_{a1}/k_{a2}$) would be unaffected. However, the structural environment of bound manganese at site $a$ would be altered, resulting in a change (decrease) in $e_{11}$.
The role of cysteine might be to initiate the reaction by providing a source of reducing equivalents for cis-aconitate. The obvious difficulties with the oxidation-reduction mechanism are the instability of metal hydrides and the unavailability of hydroxyl cations in aqueous media. Protection of these reactive intermediates from the solvent on the enzyme surface is unlikely since the hydroxyl cation must exchange with the solvent to fit the isotopic data (8) and the bound Fe(II) retains protons exchangeable with water in the ternary aconitase-iron-citrate complex, as shown by PRR (Table V). The observation that the paramagnetic susceptibility of the bound Fe(II) is unchanged in the ternary citrate complex (Table V) further argues against the formation of significant amounts of a covalent hydride complex or a π complex with the double bond of cis-aconitate, both of which would be expected to be diamagnetic (31, 32). Any mechanism involving a change in the oxidation state of Fe(II) seems unlikely. Hence, it appears that the Mn(III) complex of aconitase fails to provide the appropriate conformation for catalysis in the binary complex or in the ternary complex with cis-aconitate.

Because of its failure to activate aconitase, Mn(II) is a useful paramagnetic probe permitting binding studies with each of the three substrates separately (Table IV) and competition studies between pairs of substrates (Table VI); the active enzyme-Fe(II) complex can be titrated only with the equilibrium mixture of substrates.

Competitive substrate titrations with the Mn(II) enzyme have yielded information on the substrate specificity at each manganese site. Since the discovery of aconitase by Martius (33), many attempts have been made to determine whether all of the reactions of aconitase are catalyzed at the same site or at separate sites. The kinetic evidence has been interpreted to indicate a single catalytic center to be responsible for all of the interconversions (8, 34-37). The intramolecular proton transfer in the conversion of citrate to isocitrate provides strong support for this view (8). However, Peters and Shorthouse (7) have recently observed that partially purified aconitase preparations, on storage, selectively showed a marked decrease in the relative rate of cis-aconitate formation from citrate and isocitrate, an observation that is difficult to explain on the basis of a common site. Such data might, however, be explained by different rate-limiting steps governing the dehydration of the two substrates so that each rate might be affected differently by a structural change in the protein. Examples of such behavior have been found with aldolase (38) and with isocitrate dehydrogenase (39).

In an attempt to demonstrate whether citrate and isocitrate bind at the same metal site on aconitase, two analyzes of the data in Table VI were used. Equation 8 shows the method of obtaining the true dissociation constant for isocitrate if citrate and isocitrate both compete for E-manganese.

\[ K_{\text{1app}}^{\text{iso}} = K_1^{\text{iso}} \left[ 1 + \frac{(\text{citrate})}{K_2^{\text{cit}}} \right] \] (8)

\[ K_{\text{1app}}^{\text{iso}} \] is the apparent dissociation constant for isocitrate in the presence of citrate, \( K_1^{\text{iso}} \) is its true dissociation constant, and \( K_2^{\text{cit}} \) is the true dissociation constant for citrate. Equation 8 predicts that the \( K_{\text{1app}}^{\text{iso}} \) will increase linearly with citrate concentration. The end point of the titration, \( \epsilon_T \), would remain the same since the single site would be saturated by citrate and isocitrate.

An alternative analysis involving a specific site for isocitrate and a specific site for citrate would predict no competition between citrate and isocitrate for E-manganese and the concentration of isocitrate needed to half-saturate the binary E-manganese complex, [S], would remain constant in the presence of citrate (Table VI, Column 3). The end point, \( \epsilon_T \), would be dependent upon the amount of citrate present since \( \epsilon_T \) would be a weighted average of the enhancement for the two different sites (Table VI, Column 3). The competition between citrate and isocitrate for the enzyme-bound Mn(II) observed in the present direct binding studies and the agreement of the dissociation constants of citrate and isocitrate with their respective \( K_m \) values strongly support the hypothesis of a common site for the dehydration of both substrates. The stoichiometry for Mn(II) and for Fe(II) binding suggests the presence of two such sites per enzyme molecule.

The de-enhancement by substrates of the effect of the aconitase-Mn(II) complex on the relaxation rate of water protons (Table IV) provides suggestive evidence for the existence of enzyme-metal-substrate bridge complexes, as had been proposed for a number of enzymes (2).

The small increase in \( \epsilon^* \) on adding saturating levels of citrate to the aconitase-Fe(II) complex does not rule out an enzyme-Fe(II)-substrate bridge complex since factors other than the coordination number of iron for water influence the relaxivity (2). Thus, an increase in the water exchange rate on Fe(II), in the correlation time for Fe(II)-water interaction, or in the outer sphere contribution to the relaxivity, could explain this observation. The last effect has been observed with aldolase-manganese-substrate bridge complexes (40). Direct evidence for metal bridge complexes, which has been obtained by studies of the paramagnetic effect of the aconitase-manganese complex on the relaxation rates of the substrate citrate and the competitive inhibitor trans-aconitate, will be the subject of the next paper in this series.

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