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} = 620 ± 80 μM. 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 ± 6 μM. 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 tertiary enzyme-metal-substrate complexes. The dissociation constants (K_diss) of these tertiary Mn(II) complexes agree with the respective K_m values of citrate and isocitrate, but not of cis-aconitate using the Fe(II) enzyme. Citrate in proportion to its concentration raises the K_diss for E-manganese-isocitrate, consistent with competition of the two substrates for the same Mn(II) site on the enzyme.

Aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3] catalyzes the dehydration of both citric and isocitric acids to form cis-aconitic acid, the reverse reactions, and the interconversion of citric and isocitric acids:

\[
\text{Citrate} \xrightarrow{\text{Aconitase}} \text{Isocitrate}
\]

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).

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-D(+)-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\(^1\) at 24.3 MHz.

**Methods**

**Enzyme Assays—Aconitase** was purified to homogeneity as previously described (6). The specific activity of the enzyme was 9 \(\mu\)moles 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 (46). 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-\(\mu\)l 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 \(\mu\)l) was contained in a quartz capillary tube maintained at 25\(^\circ\) 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, \(e^o\), 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 isocitrate aconitase; 0, 0.48, and 0.90 mM Fe(NH\(_4\))\(_2\)SO\(_4\); 0, 4.8, and 9.1 mM cysteine; 11 mM tetramethylammonium chloride; and 0.1 mM Fe(NH\(_4\))\(_2\)SO\(_4\), pH 7.5, and 10 mM cysteine, pH 7.5 (when present), in a final volume of 0.1 ml (6). The solution was incubated for 30 min, and a 20-\(\mu\)l aliquot was removed and assayed for activity by means of the 340 nm assay described above with citrate as the substrate (8).

**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 (5 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 1 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 hinders 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 conformation 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.**

\[
\frac{(Mn)_{H2} + (Mn)_{H2}}{(E)_{R}(Mn)_{R}} = \frac{n_1 + n_2}{K_1} + \frac{(n_1 + n_2)(Mn)_{R}}{K_1K_2} - \frac{1}{K_1} + \frac{1}{K_2} + \frac{(Mn)_{R}}{K_1K_2} \left(\frac{(Mn)_{H2} + (Mn)_{H2}}{(E)_{R}}\right)
\]

The subscripts \(f\) and \(b\) refer to free and bound Mn(II), respectively; \((Mn)_{H2}\) and \((Mn)_{H2}\) refer to Mn(II) bound at sites 1 and 2, respectively; \((E)_{R}\) 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 curve 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 6.0 ± 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 \((Mn)_{H2}/(E)_{R} = 0\) 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 \(e_{bH}\), 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 \(e_{bH} = 3.4 ± 0.6\). If the value of \(e_{bH}\) is assumed to be constant over the entire range of concentrations activation might occur. Inactivation by high concentrations of divalent cations has been discussed in a review by Eichorn (14).

Inactivation by high concentrations of divalent cations has been discussed in a review by Eichorn (14).
TABLE I
Effect of various metals on activation of aconitase

| Metal salt | Cysteine | Percentage of activity
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1.0 mM</td>
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<tr>
<td>Fe (NH₄SO₄)₂</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>MnCl₂</td>
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<td>0</td>
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<tr>
<td>CoCl₂</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Ni (NO₃)₂</td>
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<td>0</td>
</tr>
<tr>
<td>Cu (CH₃CO₂)₂</td>
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<td>0</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>CdCl₂</td>
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<td>0</td>
</tr>
<tr>
<td>CaCl₂</td>
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<tr>
<td>RuCl₃</td>
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</tr>
<tr>
<td>RhCl₃</td>
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<td>0</td>
</tr>
<tr>
<td>Gd (NO₃)₃</td>
<td>+</td>
<td>0</td>
</tr>
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</table>

* 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 (<1%).

Final concentrations of Ni(NO₃)₂ 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).

Fig. 1. A, Scatchard plot of the binding of Mn(II) to aconitase. Fifty-microliter solutions of aconitase (0.427 mM), containing from 0.02 to 2.0 mM MnCl₂ in 0.05 M triethanolamine chloride, pH 7.5, were contained in quartz capillaries and the amount of free Mn(II) was measured as described under "Methods." The temperature was 25°. The straight lines are the Scatchard plots of two sets of thermodynamically independent sites which would yield the resultant curve which fits the data. B, variation of the binary enhancement, e₂, with the amount of bound Mn(II) per total enzyme. Each solution used for the above Scatchard plot was placed in a pulsed NMR spectrometer as described under "Methods" and the longitudinal relaxation rate, 1/T₁, of the protons of water was measured. The enhancement was calculated as described in Reference 11.

TABLE II
Effect of ratio of bound Mn(II) to total enzyme on binary enhancement, e₂

<table>
<thead>
<tr>
<th>(Mn)₃</th>
<th>(Mn)₂</th>
<th>(Mn)₁</th>
<th>(Mn)₁/(E)</th>
<th>(Mn)₂/(E)</th>
<th>e₂</th>
<th>(Mn)₃/(E)</th>
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<tr>
<td>0.02</td>
<td>0.0005</td>
<td>0.07</td>
<td>0.01</td>
<td>0.09</td>
<td>3.8</td>
<td>6.7</td>
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<tr>
<td>0.04</td>
<td>0.0030</td>
<td>0.14</td>
<td>0.02</td>
<td>0.21</td>
<td>2.8</td>
<td>1.89</td>
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<tr>
<td>0.06</td>
<td>0.0047</td>
<td>0.20</td>
<td>0.04</td>
<td>0.30</td>
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<td>1.40</td>
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<td>0.10</td>
<td>0.0081</td>
<td>0.32</td>
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<td>0.50</td>
<td>2.7</td>
<td>1.44</td>
</tr>
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<td>0.15</td>
<td>0.0130</td>
<td>0.40</td>
<td>0.10</td>
<td>0.70</td>
<td>2.4</td>
<td>0.99</td>
</tr>
<tr>
<td>0.20</td>
<td>0.0178</td>
<td>0.62</td>
<td>0.17</td>
<td>0.90</td>
<td>2.5</td>
<td>0.70</td>
</tr>
<tr>
<td>0.30</td>
<td>0.0330</td>
<td>0.88</td>
<td>0.29</td>
<td>1.10</td>
<td>2.2</td>
<td>0.06</td>
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<tr>
<td>0.40</td>
<td>0.0520</td>
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<td>0.43</td>
<td>1.30</td>
<td>1.9</td>
<td>0.24</td>
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<tr>
<td>0.60</td>
<td>0.0941</td>
<td>1.45</td>
<td>0.76</td>
<td>1.60</td>
<td>1.6</td>
<td>0.34</td>
</tr>
<tr>
<td>1.00</td>
<td>0.228</td>
<td>1.86</td>
<td>1.01</td>
<td>1.84</td>
<td>1.5</td>
<td>0.69</td>
</tr>
<tr>
<td>2.00</td>
<td>0.800</td>
<td>2.08</td>
<td>3.18</td>
<td>3.58</td>
<td>1.7</td>
<td>0.90</td>
</tr>
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</table>

* This atypically high value was ignored in determining the limiting value of e₂ at (Mn)₃/(E) = 0.
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 \( K_d^{Fe} \) is 0.39 ± 0.10 mM, which was determined separately by PRR. (c) 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. (d) The value of \( e^* \) at the end point (1.4 ± 0.1) was independent of aconitase concentration over a 3.5-fold range from 0.40–1.38 mM aconitase sites. 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.

\[
K_4 = \frac{\text{aconitase-manganese}}{\text{aconitase}} \times \frac{\text{citrate}}{\text{(冷漠)}} = 0.39 ± 0.10 \text{ mM (4)}
\]

The enhancement of the ternary complex (\( e_4 \)) 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

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### Table III

<table>
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<tr>
<th>(Mn)(/E)</th>
<th>Fe</th>
<th>Mn</th>
<th>K_d^Fe</th>
<th>K_d^Fe</th>
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<tr>
<td>0.38</td>
<td>2.8</td>
<td>0.125</td>
<td>0.200</td>
<td>0.049</td>
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<tr>
<td>0.18</td>
<td>2.6</td>
<td>0.875</td>
<td>0.200</td>
<td>0.006</td>
</tr>
<tr>
<td>0.14</td>
<td>2.7</td>
<td>0.988</td>
<td>0.200</td>
<td>0.100</td>
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<tr>
<td>0.13</td>
<td>2.1</td>
<td>0.125</td>
<td>0.200</td>
<td>0.105</td>
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<tr>
<td>0.09</td>
<td>2.2</td>
<td>0.250</td>
<td>0.200</td>
<td>0.107</td>
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<tr>
<td>0.12</td>
<td>1.0</td>
<td>0.370</td>
<td>0.185</td>
<td>0.039</td>
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<tr>
<td>1.13</td>
<td>0.620</td>
<td>0.758</td>
<td>0.230</td>
<td>0.497</td>
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<tr>
<td>0.77</td>
<td>1.244</td>
<td>0.685</td>
<td>0.190</td>
<td>0.103</td>
</tr>
</tbody>
</table>

Average: 0.035 ± 0.023, 0.016 ± 0.006.

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

* 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 mM triethanolamine chloride, pH 7.5, was titrated with an identical solution but which also contained 3.5 mM 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. Consistent with the formation of aconitase-manganese-substrate bridge complexes in which water ligands on the enzyme-bound citrate and cis-aconitate compete for the same manganese site, the et values of 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-citrate 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 Ks of cis-aconitate (0.53 mM) is still more than an order of magnitude greater than the Ks of citrate-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/\(T_1\)) of Fe(II) is decreased by ~60% in the aconitase-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 ~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 relaxivity remains constant. The paramagnetic susceptibility does not change from that of the binary complex. The paramagnetic relaxivity of the binary aconitase-Fe(II) complex and the ternary aconitase-Fe(II)-substrate complex (90% citrate at equilibrium (21)) indicates that Fe(II) remains ~60% paramagnetic (high spin) in the presence of enzyme, citrate, and substrates.

Michaelis Constants of Substrates of Aconitase—The Michaelis constants (Km) of the three substrates of Fe(II)-activated aconitase were determined for the homogenous aconitase preparation and are given in Table IV. The present Km 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 substrates of aconitase from the aconitase-Mn(II)-substrate complex (Ks) with the respective Km values of the substrates with Fe(II)-activated aconitase. The Ks values for citrate and isocitrate are in good agreement with their Km values, but the Ks value for cis-aconitate is two orders of magnitude greater than its Km, suggesting a different mode of binding of cis-aconitate to the Fe(II) and Mn(II) complexes of the enzyme.

Comparison Between Citrate and Isoctirate 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

### Table IV

<table>
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<tr>
<th>Substrate</th>
<th>(K_b)</th>
<th>(K_m)</th>
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<tbody>
<tr>
<td>Citrate</td>
<td>1.4</td>
<td>0.39 ± 0.1</td>
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<tr>
<td>Isoctirate</td>
<td>1.5</td>
<td>0.57 ± 0.1</td>
</tr>
<tr>
<td>cis-Aconitate</td>
<td>1.6</td>
<td>0.53 ± 0.1</td>
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</table>

*a* In a typical experiment, a 50-μliter solution of aconitase, 1.10 mM active sites, and MnCl₂ (144 μM) in 0.05 M triethanolamine chloride, pH 7.5, was titrated with an identical solution, which also contained either 3.5 mM potassium citrate, 3.75 mM potassium isocitrate, or 10 mM potassium cis-aconitate. The 1/\(T_1\) of water protons was measured as described under “Methods.” The temperature was 22°C.

*b* 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. Km values were determined at four to five concentrations of each substrate over a 20- to 50-fold range, including the Km by double reciprocal plots.

### Table V

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fe(II)</th>
<th>Aconitase</th>
<th>Citrate</th>
<th>Cysteine</th>
<th>(1/T_1) (µs⁻¹)</th>
</tr>
</thead>
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<td>A</td>
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<td></td>
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*a* These values were corrected for the water relaxation due to the bound Fe(II) of aconitase (6).
In the competition experiment between Fe(II) and Mn(II), the data were fit by a simplifying assumption 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). The alternative assumption of thermodynamic site-site interaction might have been used to fit the Fe(II) binding data. However, this more complicated assumption, although not excluded, is clearly not required to fit the data (Table III, last column).

Aconitase has been found to be activated only by Fe(II), and it appears that a reducing agent is necessary to achieve maximum activity, possibly by keeping iron in the divalent oxidation state at pH 7.5. Although the absolute specificity for Fe(II) in the activation of aconitase remains unexplained, the present work provides some information on this point. Some of the metal ions shown in Table I may fail to activate aconitase because they do not bind at the active site or because they inhibit by binding at other sites. Such an explanation is unlikely for Mn(II), which occupies the same sites as Fe(II) does, as determined by the competitive binding study (Table III). Moreover, the enzyme-manganese complex forms ternary complexes with each of the substrates of aconitase (Fig 3; Table IV). Four explanations for the failure of Mn(II) to activate the enzyme may be considered. (a) The previously demonstrated slow and stepwise activation of aconitase by Fe(II) and cysteine (6) suggests that site-site interaction occurs which lowers the enzyme activity, possibly by keeping iron in the divalent oxidation state at pH 7.5. Although the absolute specificity for Fe(II) in the activation of aconitase remains unexplained, the present work provides some information on this point. Some of the metal ions shown in Table I may fail to activate aconitase because they do not bind at the active site or because they inhibit by binding at other sites. Such an explanation is unlikely for Mn(II), which occupies the same sites as Fe(II) does, as determined by the competitive binding study (Table III). Moreover, the enzyme-manganese complex forms ternary complexes with each of the substrates of aconitase (Fig 3; Table IV). Four explanations for the failure of Mn(II) to activate the enzyme may be considered. (a) The previously demonstrated slow and stepwise activation of aconitase by Fe(II) and cysteine (6)

\[ \text{Fe(II)} + \text{H}_{2}\text{O} \rightarrow \text{Fe}^{3+} + 2\text{H}^{+} \]

and the partial inhibition of this process by excess Mn(II) suggests that the participation of complicated conformation alterations in the protein, possibly involving oxidation-reduction reactions, as has been observed with Taka-amylase (27) and deoxyribonuclease (28). Divalent Mn(II) would be less likely than iron to participate in such processes. (b) The decrease in the paramagnetic susceptibility of Fe(II), when bound to aconitase, is consistent with the formation of ~42% low spin Fe(II) in the active complex. Divalent manganese generally remains high spin in its complexes, whereas divalent iron can rapidly change its spin state between high and low spin as occurs in the liganding reactions of deoxyhemoglobin (29, 30). (c) The low affinity of cis-aconitate for the aconitase Mn(II) complex, as compared to its K_m with the Fe(II)-activated enzyme (Table IV), suggests that the manganese-enzyme complex may not bind this substrate properly. Since bound cis-aconitate participates in all of the reactions of aconitase, the improper binding of this substrate could prevent catalysis. (d) The catalytic reaction itself may involve an oxidation-reduction process,
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 \( \pi \) 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(II) 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-Mn(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 analyzers 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.

\[
K_{\text{iso}}^{\text{iso}} = K_3^{\text{iso}} \left[ 1 + \frac{[\text{citrate}]}{K_3^{\text{cit}}} \right]
\]  

(8)

\( K_{\text{iso}}^{\text{iso}} \) is the apparent dissociation constant for isocitrate in the presence of citrate, \( K_3^{\text{iso}} \) is its true dissociation constant, and \( K_3^{\text{cit}} \) is the true dissociation constant for citrate. Equation 8 predicts that the \( K_3^{\text{iso}} \) will increase linearly with citrate concentration. The end point of the titration, \( \epsilon_f \), 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, \([S]_o\), would remain constant in the presence of citrate (Table VI, Column 3). The end point, \( \epsilon_f \), would be dependent upon the amount of citrate present since \( \epsilon_f \) would be a weighted average of the enhancements 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 aconitate-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 aconitate-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 aconitate-manganese complex on the relaxation rates of the substrate citrate and the competitive inhibitor \( \text{trans}-\text{aconitate} \), will be the subject of the next paper in this series.

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