The Number of Substrate- and Inhibitor-binding Sites of Fumarase*

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SUMMARY

The reversible binding to fumarase of the competitive inhibitors, trans-aconitate and citrate, and of the natural substrates, fumarate and L-malate, was studied by the method of equilibrium dialysis. The binding of the enol tautomer of oxalacetate, a secondary substrate of fumarase, was measured spectrophotometrically. The results of these studies indicate that there are four substrate- or inhibitor-binding sites per tetrameric molecule of enzyme, or an average of one site per polypeptide chain subunit. The Michaelis constants ($K_m$) and inhibition constants ($K_i$) for these compounds were found to be in close agreement with the dissociation constants calculated from the binding studies. This correspondence strongly suggests that the four binding sites measured are also catalytic sites.

Earlier studies have shown that fumarase (fumarate hydratase, EC 4.2.1.2) from swine heart muscle is composed of four identical, subunit polypeptide chains (1). The chains are held together by noncovalent interactions to form the enzymatically active, tetrameric molecule (1). Because dissociation of the subunits by a variety of means leads to a complete loss of enzymatic activity (2), it was of interest to determine the number of active sites per tetrameric molecule of enzyme. We wish to report here the results of studies on the reversible binding of fumarate, L-malate, and competitive inhibitors to fumarase. The binding of these compounds has been measured principally by the method of equilibrium dialysis. The results of these studies indicate that there are four binding sites per molecule of enzyme, or an average of one per polypeptide chain subunit. Because the dissociation constants of the enzyme and each of the compounds correspond closely with the kinetically determined Michaelis constants ($K_m$) or inhibition constants ($K_i$) of the compounds, these studies suggest that the four binding sites are equivalent to the active sites of fumarase.

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EXPERIMENTAL PROCEDURE

Materials

Fumaric-2,3-14C acid was purchased from the International Chemical and Nuclear Corporation and citric-1,5-14C acid from New England Nuclear. Grade A L-malic, fumaric, trans-aconitic, and oxalacetic acids were obtained from Calbiochem, and sodium citrate from J. T. Baker Chemical Company, Phillipsburg, New Jersey. DPNH was purchased from Sigma.

Scintillation solution was prepared from 1 part Triton X-100 and 2 parts toluene-2,5-diphenyloxazole (PPO)-1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethylPOPOP) solution (3). The latter two reagents were purchased from the Packard Instrument Company. All other reagents were of the highest purity commercially available.

Methods

Analytical Methods—Optical densities were measured on a Cary 15 recording spectrophotometer and a Zeiss PMQ II spectrophotometer. A Packard Tri-Carb model 314-EX scintillation counter was used to count 14C-labeled samples. The pH of solutions was measured with a Radiometer model PHM 22 pH meter equipped with a GK2021C combined electrode.

Enzymes—Fumarase, prepared from swine heart muscle by the method of Kanarek and Hill (4), was stored at 5°C as a crystalline suspension in 55% ammonium sulfate. Solutions of enzyme were prepared from the crystals as described earlier (5). All enzyme solutions were dialyzed for approximately 2 hours against a 1,000-fold excess of the desired buffer and finally passed through a 0.8-μ Millipore filter to remove the last traces of undissolved material. Fumarase prepared in this manner had a specific activity of 32,000 to 34,000 units per mg when assayed as described previously (4). Fumarase concentrations were determined spectrophotometrically at 280 mp, where a 0.1% solution at pH 7.3 has an absorbance of 0.51.

Malic dehydrogenase, purchased from Continental Biochemical Corporation as a crystalline suspension in NH4SO4, was used without further purification. The enzyme was solubilized by dialysis against 0.01 M Tris-acetate buffer, pH 7.3.

Equilibrium Dialysis—These studies were performed with dialysis cells similar to those described earlier (6). The cells, constructed from Plexiglas, contained two circular chambers...
Control experiments were performed by adding solutions of trans-aconitate or $^{14}$C-fumarate to one dialysis chamber and buffer to the other. The concentration of these compounds in both dialysis chambers was determined at 1-hour intervals after dialysis was initiated. The results of these control experiments indicated that equilibrium was established within 8 hours and that ion binding to the dialysis membrane was sufficiently small that it could not be detected within the experimental error of the technique.

The binding values determined from equilibrium dialysis measurements were corrected for Donnan effects in the following manner.

1. Dialysis solutions were considered to contain only monovalent ion at the total molar concentration of all ion species present. Thus, for example, a solution containing 0.1 M sodium acetate, 0.01 M sodium phosphate, and 0.002 M sodium citrate was considered to contain only 0.10 M sodium acetate for the purposes of calculation. Since dialysis solutions normally contained a large excess of sodium acetate (see “Results”), the error introduced by this approximation had a negligible effect on final binding values.

2. The net charge on the fumarase molecule at pH 7.6 in the presence of phosphate and buffer was estimated to be $-10$ from electrophoretic mobility measurements (7). In the presence of substrate or competitive inhibitors, the negative charge on the molecule was considered to increase proportionally to the amount of the anions bound.

3. From 1 and 2, the concentration of monovalent ion on each side of the dialysis membrane due to the Donnan effect was calculated in the usual manner (8).

4. Because the concentrations of the di- and trivalent binding anions (fumarate-malate, citrate, and trans-aconitate) were small relative to the concentration of acetate, the distribution of the former ions across the membrane could be calculated from the relation (8)

$$
\frac{X}{X'} = \frac{Y}{Y'} = \frac{Z}{Z'}
$$

where

$$
\frac{X}{X'} = \text{ratio of monovalent ion determined in Step 3}
$$

$$
\frac{Y}{Y'} = \text{ratio of divalent ion}
$$

$$
\frac{Z}{Z'} = \text{ratio of trivalent ion}
$$

From the above ratios and knowledge of the total concentrations of the di- and trivalent anions, the absolute concentration of these ions in each dialysis chamber due to the Donnan effect was calculated and corrected for.

**Kinetic Measurements**—The initial rate of dehydration of L-malate by fumarase was measured spectrophotometrically between 220 and 270 mU. Aliquots (5-$\mu$l) of fumarase were added to 3-$ml$ solutions of L-malate and the increase in optical density, due to the formation of fumarate, was monitored at 10-sec intervals. The extinction coefficients of fumarate used for these kinetic measurements were those determined by Albert et al. (9).

Inhibition constants ($K_i$) were determined by measuring the initial rate of dehydration of L-malate ($1 \times 10^{-4} \text{ M}$ to $5 \times 10^{-4} \text{ M}$) in the absence and in the presence of one of the competitive inhibitors, trans-aconitate ($5 \times 10^{-4} \text{ M}$), oxalacetate ($1 \times 10^{-4} \text{ M}$). Inhibition by trans-aconitate and citrate was measured in the presence of 0.01 M phosphate and 0.1 M acetate at pH 7.6. Inhibition by oxalacetate was determined in 0.01 M Tris-acetate at pH 7.3. These measurements were plotted by the method of Lineweaver and Burk (10), and $K_i$ was evaluated from the curves in the usual manner.

The concentration of oxalacetate was determined by an assay with malic dehydrogenase. Malic dehydrogenase (0.5 ml; 10,000 units), DPNH (0.5 ml; 7 $\times 10^{-4}$ M), and a stock solution of oxalacetate (0.1 ml; $\sim 1.5 \times 10^{-4}$ M) were added into a 3-$ml$ cuvette. Oxidation of DPNH by oxalacetate was monitored at 340 mU, where $A_{DPNH} = 6.22 \times 10^3$ (11). From the equilibrium constant for this reaction (12) it has been calculated that $>99.9\%$ of the oxalacetate is reduced under these assay conditions.

**RESULTS**

**Equilibrium Dialysis**

The reversible binding of the competitive inhibitors, trans-aconitate and citrate, and of the natural substrates, fumarate and L-malate, to fumarase was studied by the method of equilibrium dialysis.

**Trans-aconitate**—Concentrations of trans-aconitate ranging from $2 \times 10^{-4}$ M to $1.6 \times 10^{-3}$ M were equilibrated at $25^\circ \pm 1^\circ$ with $3.9 \times 10^{-4}$ M fumarase solutions as described in “Methods.” All solutions were buffered in 0.1 M sodium acetate and 0.001 M sodium phosphate at pH 7.6. The binding of trans-aconitate was determined by measuring the difference in concentration between the trans-aconitate solution initially added to the dialysis chamber, $[\text{trans-aconitate}]_0$, and the $[\text{trans-aconitate}]$ solution removed from the same chamber after equilibrium was established, $[\text{trans-aconitate}]_e$.

Trans-aconitate concentrations were determined spectrophotometrically by measuring the absorbance of these solutions between 230 and 290 mU. The experimentally determined extinction coefficients of trans-aconitate listed below were used for these calculations.

<table>
<thead>
<tr>
<th>$\lambda$ (mU)</th>
<th>$\epsilon$ (M$^{-1}$ cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>230</td>
<td>$5.00 \times 10^4$</td>
</tr>
<tr>
<td>240</td>
<td>$4.21 \times 10^4$</td>
</tr>
<tr>
<td>250</td>
<td>$2.47 \times 10^4$</td>
</tr>
<tr>
<td>260</td>
<td>$1.25 \times 10^4$</td>
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The equilibrium concentrations of trans-aconitate were corrected for the Donnan effects (see “Methods”) and $r$, the moles...
of trans-aconitate bound per mole of enzyme, was calculated from the following expression.

\[
\tilde{v} = \frac{[\text{trans-aconitate}]_{\text{obs}} - 2[\text{trans-aconitate}]_{\text{eq}}}{[\text{fumarase}]}
\]

The moles of ion bound per mole of enzyme, \(\tilde{v}\), was determined at several different concentrations of trans-aconitate, and plotted with the Scatchard equation (13)

\[
K_{eq} \frac{\tilde{v}}{C} = -\tilde{v} + n
\]

where \(C\) is the concentration of unbound ion in equilibrium with fumarase and bound ion, \(n\) is the number of ion-binding sites per molecule of enzyme, and \(K_{eq}\) is the dissociation constant for the enzyme-ion complex.

A plot of \(v/C\) versus \(v\) for trans-aconitate is shown in Fig. 1A. Extrapolation of this binding curve to saturating concentrations of trans-aconitate \((v/C \to 0)\) gives a value for \(n\) of 4.0.

**Citrate**—Solutions of sodium citrate-1,5-\(^{14}\)C (1 mC/30 mmoles) ranging in concentration from 5 \(\times 10^{-4}\) M to 1.5 \(\times 10^{-2}\) M were equilibrated at 25° \(\pm 1^\circ\) with 6.1 \(\times 10^{-3}\) M fumarase solutions. All solutions were buffered in 0.05 M sodium acetate and 0.01 M sodium phosphate at pH 7.6. After equilibrium was established, the concentration of \(^14\)C-citrate in both dialysis chambers was determined by measuring the radioactivity of these solutions. An aliquot (500 \(\mu\)l) from each chamber was mixed with 10 ml of scintillation solution and then counted for 5 min. The equilibrium concentrations of citrate were corrected for the Doman effect as described earlier. The moles of ion bound per mole of enzyme, \(\tilde{v}\), was calculated from the expression

\[
\tilde{v} = \frac{[\text{citrate}]^A - [\text{citrate}]^B}{[\text{fumarase}]}
\]

where the superscript \(A\) refers to the chamber containing fumarase and \(B\), the chamber containing only citrate. The binding of citrate at several different concentrations was measured and expressed as a Scatchard plot, as shown in Fig. 1B. The binding curve extrapolates to a value of 3.8 binding sites for citrate.

**Fumarate-Malate**—Solutions (1.2 \(\times 10^{-4}\) M to 3.6 \(\times 10^{-4}\) M) of sodium fumarate-2,3-\(^{14}\)C (1 mC/10 mmoles) were dialyzed at 25° \(\pm 1^\circ\) against 3.8 \(\times 10^{-4}\) M fumarase solutions. All solutions were buffered in 0.1 M sodium acetate and 0.01 M sodium phosphate at pH 7.6. Because the rate of the enzymatic equilibrium of fumarate and l-malate is appreciably greater than the rate of diffusion of these compounds across the dialysis membrane, it was assumed that an equilibrium mixture (4.4 moles of l-malate per mole of fumarate) of the substrates existed in both chambers when dialysis was complete. The \(v\) values for these compounds were measured as described for the \(^14\)C-citrate binding experiments. A Scatchard plot of the fumarate-malate data is shown in Fig. 1C. A value of 3.6 binding sites per molecule of fumarase was calculated from the binding of the substrate mixture.

**Binding of Oxalacetate**

Oxalacetate is the product of the fumarase-catalyzed hydration of acetylenedicarboxylate and the dehydration of L-tartrate (14), and may be considered to be a substrate of fumarase. In practice, however, oxalacetate behaves only as a competitive inhibitor of the enzyme, since the formation of oxalacetate by these reactions appears to be essentially irreversible.

The binding of oxalacetate to fumarase was determined by measuring the amount of the enol tautomer bound to the enzyme. Fumarase (2 ml; 6.85 mg per ml) was added to 1.0 ml of oxalacetate at concentrations over a range from 6.1 \(\times 10^{-4}\) M to 1.6 \(\times 10^{-3}\) M. All solutions were buffered in 0.01 M Tris-acetate, pH 7.3. The absorbance of the fumarase-oxalacetate mixtures was measured at 255 nm against a blank solution containing fumarase at the same concentration. The absorbance values measured were greater than could be accounted for by the known concentration of oxalacetate in tautomeric equilibrium. The increased absorbance was therefore attributed to the preferential binding of the enol tautomer to fumarase, since this species has an extinction coefficient which is appreciably greater than that of the keto form at 255 nm. The binding of the enol tautomer was quantitatively measured with the equation

\[
A_{\text{total}} = [\text{enol}]_{\text{bound}} \times 1.24 \times 10^3 + ([\text{OAA}]_{\text{total}} - [\text{enol}]_{\text{bound}}) \times 7.36 \times 10^3
\]

where OAA is oxalacetate.

The extinction coefficient of oxalacetate was determined by measuring the absorption of a solution of oxalacetate in tautomeric equilibrium at pH 7.3. The concentration of this solution was determined where the aid of malic dehydrogenase as described in "Methods." The extinction coefficient measured is in close agreement with value earlier published (15). The extinction coefficient of the enol tautomer was calculated from the value for the tautomeric mixture and the fact that approximately 85% of oxalacetate at pH 7.3 exists as the keto tautomer (16, 17). The extinction coefficient of the keto tautomer used in the above
calculation was estimated to be 160 from the absorbance of \( \alpha,\alpha \)-dimethyloxalacetate, which does not exist in the enol form (18). The number of moles of enol tautomer bound per mole of fumarase was measured at several concentrations of oxalacetate and the results were plotted by the method of Scatchard, as shown in Fig. 1D. A value of 3.9 binding sites per molecule was determined from these measurements.

**Dissociation Constants**

Equilibrium constants for the dissociation of the fumarase-substrate and fumarase-inhibitor complexes, as determined from the slopes of the binding curves in Fig. 1, are listed in Table I. Also presented in Table I, for comparison, are the average Michaelis constants for an equilibrium mixture of fumarate and L-malate and the inhibition constants for trans-aconitate, citrate, and oxalacetate. The average \( K_m \) value was calculated from the equation

\[
K_{m(\text{avg})} = \frac{5.4}{\frac{1}{K_F} + \frac{1}{K_M}}
\]

where \( K_F \) and \( K_M \) are the Michaelis constants for fumarate and L-malate, respectively.

The \( K_m \) values of fumarate and L-malate used in this calculation were measured by Alberty et al. (9). \( K_F \) values were calculated from Lineweaver-Burk plots of competitive inhibition experiments as described in "Methods."

**DISCUSSION**

The binding curves shown in Fig. 1 give values of \( n \) which range from 3.6 for the fumarate-malate mixture to 4.0 for trans-aconitate. It has been concluded from these data that there are four substrate-binding sites per tetrameric unit of fumarase, or an average of one site per polypeptide chain subunit. This finding is consistent with the earlier estimate by Shavit, Wolfe, and Alberty that there are a maximum of 5 + 2 catalytic sites per molecule of molecular weight 194,000 (7). This estimate was calculated from kinetic data, which indicate that there are 2 groups at the enzymatic site with a \( pK \) equal to 7.4, and from the titration curves, which reveal that there are a total of 10 + 2 such groups per molecule of enzyme.

Although it cannot be demonstrated unequivocally that all four substrate-binding sites are catalytic sites, it is apparent that these sites possess properties in common with the catalytic sites.

1. All four sites have an affinity for both hydrated and unhydrated species, an essential property of the active sites of fumarase. It was observed that both trans-aconitate, which is an analogue of fumarate, and citrate, which is an analogue of L-malate, bind at four sites. This observation, together with the finding that the fumarate-malate mixture was also bound at only four sites, indicate that hydrated and unhydrated species are bound at the same site.

2. The four binding sites and the catalytic sites have an approximately equal affinity for substrates and competitive inhibitors. It may be seen in Table I that the \( K_m \) and \( K_F \) values, which are a measure of the affinity of substrate or competitive inhibitors for the active site, are in close agreement with the corresponding dissociation constants, measured by binding experiments. The finding that \( K_{eq} \) and \( K_m(\text{avg}) \) for the fumarate-malate mixture are nearly equal is consistent with the kinetic measurements of Alberty and Pierce, who showed that the \( K_m \) value of fumarate, within experimental error, is equal to the enzyme-substrate dissociation constants for this compound (19).

The correspondence between the kinetic and binding constants also indicates that the affinity of fumarase for its substrate and competitive inhibitors remains unchanged over a wide range of enzyme concentrations. This conclusion was reached because the fumarase solutions used to measure binding were approximately 100,000 times more concentrated than those used for the kinetic measurements.

The kinetic constants in Table I indicate that oxalacetate has a greater affinity for fumarase than the natural substrates, fumarate or L-malate. The relatively low dissociation constants measured for oxalacetate, however, can be attributed to the fact that the binding of this compound was measured in the absence of phosphate buffer, a competitive inhibitor of the enzyme. \( K_m \) values for fumarate and L-malate measured in 0.005 M Tris-acetate at pH 7.0 are correspondingly low (20).

The finding that fumarase possesses four substrate-binding sites is consistent with the fact that the enzyme is composed of four identical polypeptide chain subunits. It has not been established, however, whether each site is formed from the amino acid residues of a single polypeptide chain or from the residues of two or more identical chains. It has been observed that the tetrameric molecule of native fumarase may be dissociated by thiol reagents (2) and at acidic \( \text{pH} \) into smaller subunits. These subunits formed at acidic \( \text{pH} \) are conformationally very similar to the native protein as judged by optical rotatory dispersion measurements, but are catalytically inactive. Although these observations might suggest that the catalytic sites of fumarase are located between subunits, it is more likely that these species are inactive because of small conformational changes within the subunits themselves.

**REFERENCES**


\(^1\) J. W. Teipel and R. L. Hill, unpublished observations.


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