Pig Heart Fumarase Contains Two Distinct Substrate-binding Sites Differing in Affinity

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A eukaryotic fumarase is for the first time unequivocally shown to contain two distinct substrate-binding sites. Pig heart fumarase is a tetrameric enzyme consisting of four identical subunits of 50 kDa each. Besides the true substrates L-malate and fumarate, the active sites (sites A) also bind their analogs D-malate and oxaloacetate, as well as the competitive inhibitor glycine. The additional binding sites (sites B) on the other hand also bind the substrates and their analogs D-malate and oxaloacetate, as well as L-aspartate which is not an inhibitor. Depending on the pH, the affinity of sites B for ligands (K$_d$ being in the millimolar range) is 1–2 orders of magnitude lower than the affinity of sites A (of which K$_d$ is in the micromolar range). However, saturating sites B results in an increase in the overall activity of the enzyme. The benzenetetracarboxyl compound pyromellitic acid displays very special properties. One molecule of this ligand is indeed able to bind into a site A and a site B at the same time. Four molecules of pyromellitic acid were found to bind per molecule fumarase, and the affinity of the enzyme for this ligand is very high (K$_d$ = 0.6 to 2.2 µM, depending on the pH). Experiments with this ligand turned out to be crucial in order to explain the results obtained. An essential tyrosine residue is found to be located in site A, whereas an essential methionine residue resides in or near site B. Upon limited proteolysis, a peptide of about 4 kDa is initially removed, probably at the C-terminal side; this degradation results in inactivation of the enzyme. Small local conformational changes in the enzyme are picked up by circular dichroism measurements in the near-UV region. This spectrum is built up of two tryptophanyl triplets, the first one of which is modified upon saturating the active sites (A), and the second one upon saturating the low affinity binding sites (B).

Fumarase (fumarate hydratase, EC 4.2.1.2) catalyzes the reversible, stereospecific addition of water to fumarate to form L-malate (1). Being an enzyme of the citric acid cycle, it serves as an entry point of the cytoplasmic enzyme that is encoded by the same gene (2–4). These fumarase molecules are tetramers consisting of identical subunits of 50 kDa each, and their activity does not depend upon the presence of metal cations (1). In prokaryotes on the other hand, there are two distinct classes of fumarase molecules: class I fumarases are heat-labile and Fe$^{2+}$-dependent, dimeric enzymes with subunits of 60 kDa each, having no obvious sequence homology to the eukaryotic enzymes, whereas class II fumarases are heat-stable, Fe$^{2+}$-independent tetrameric enzymes with subunits of 50 kDa and showing extensive homology to the eukaryotic fumarases. Recently the three-dimensional structure of Escherichia coli class II fumarase has been unraveled (5–7). According to crystallographic data, each subunit is composed of three domains, of which the central one (D2) forms a 5-helix bundle. The association of the D2 domains was shown to result in tetramer formation, domains D1 and D3 capping at opposite ends stabilizes the active site (10).

The kinetics of the reaction catalyzed by pig heart fumarase have been studied in the past by several authors and have been summarized by Alberts (11) and Hill and Teipel (1). These investigations showed that, whereas at low substrate concentrations the Michaelis-Menten kinetics giving classic saturation curves in the $v$ versus $[S]$ plots, at substrate concentrations higher than 5 × $K_m$, substrate activation occurs, and at substrate concentrations above 0.1 M inhibition is observed. The latter is explained by the binding of two substrate molecules at the same time into the enzyme's active site, thereby forming non-efficient enzyme-substrate complexes. Several mechanisms can be put forward to explain substrate activation. (a) The enzyme preparation might either contain two different fumarase species or two non-convertible conformational forms of a single species, each enzyme form obeying classical Michaelis-Menten kinetics and having its own set of $K_m$ and $V_{max}$ values. No indications for heterogeneity can, however, be detected through electrophoretic or physicochemical analyses (12, 13). Moreover, the existence of distinct but stable conformers is highly improbable since denaturation as well as enzyme modification processes progress through simple kinetics (see e.g. Refs. 14 and 15). (b) Another mechanism is that of negative cooperativity. According to this model, binding of the first molecule of substrate into one of the four, initially similar binding sites decreases the affinity for substrate mole-
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The recycling of the enzyme involves at least two proton transfer steps through various conformational states differing in substrate specificity and catalytic activity. In their model, subunits of the enzyme’s catalytic site, which is supposed to be associated to residues Ser-98, Thr-100, Ser-139, Ser-140, and Asn-141 from the b-subunit, and His-188 from the d-subunit (6, 7). An additional binding site (site B) is supposed to be nearby and is built up of residues belonging to one single subunit (the b-subunit), i.e. Arg-126, His-129, Asn-131, and Asp-132 (5). Conclusive evidence that site A is indeed the enzyme’s catalytic site was provided by site-directed mutagenesis experiments (7). In view of the above presented arguments, it seemed of interest to investigate substrate binding with the most well known eukaryotic fumarase from pig heart tissue. In this article we present evidence that this fumarase also possesses two functional sites on the enzyme. This hypothesis is ruled out since Teipel and Hill (16) found linear Scatchard plots from equilibrium dialysis experiments. (c) Still another mechanism explaining the activation implies the existence of additional substrate-binding sites, other than and spatially separated from the active sites. The affinity of these supplementary substrate-binding sites may be supposed to be lower, but once they are occupied, the catalytic rate at the level of the active sites is increased due to (slight) conformational changes in the enzyme. Alternatively, the second substrate-binding sites may be potential catalytic sites as well. A number of anions, especially phosphate, have also been shown to affect the activity of pig heart fumarase (1, 11, 17–19). They can be supposed to bind to the substrate-binding sites as well, thereby exerting an effect on the enzyme which is comparable to the effect induced by the substrates themselves. (d) However, more recently Rose and co-workers (20–22) suggested that activation of fumarase at high concentrations of l-malate or fumarate can be explained without assuming additional substrate-binding sites. According to their investigations with pig heart (20, 21) as well as with a yeast fumarase (22), the interconversion of substrate-and product-free so-called “isofoms” of the enzyme might be a rate-limiting step during catalysis. It was claimed that activation of fumarase at substrate concentrations above 5 × Kd can be explained solely by such a recycling of free enzyme molecules through various conformational states differing in substrate specificity and catalytic activity. In their model, substrate specificity of the isoforms will depend upon the inter-relationship between both types of sites. Moreover the dissociation constants for both types of binding sites have been determined for a whole series of substrate analogs.

Materials and Methods

Reagents—Fumarase substrates and inhibitors were preanalysis grade and purchased from Sigma, as well as benzylbromide and the proteases trypsin (TPCK1-treated) and subtilisin. All benzenecarboxylic acids were from Aldrich; pyrrolidemetic acid was recrystallized from distilled water. Tetranitromethane was from Fluka AG. Solutions of urea were deionized immediately before use by passing them through a column of Amberlite MB-3 resin (Rohn and Haas Co.), and the concentration was estimated from the refractive index of the deionized solutions (23). Acrylamide and N,N'-methylene bisacrylamide were obtained from Fluka AG; they were recrystallized, respectively, from acetone and chloroform.

Fumarase—Fumarase was purified to homogeneity from pig heart tissue either by the procedure of Rabin and Hase (24), as described by Beeckmans and Kanarek (24). Enzyme solutions were prepared by dialyzing an appropriate amount of ammonium sulfate suspension against 10 mM potassium phosphate buffer, pH 7.0, until the crystals were dissolved, and then against the buffer of choice.

Routinely, fumarase activity is measured spectrophotometrically with l-malate as substrate (50 mM l-malate in 50 mM potassium phosphate buffer, pH 7.9) as described before (12). One unit of activity is the amount of enzyme which catalyzes the formation of 1 µmol of fumarate per min at 25°C; measurements are performed at 250 nm where εfumarate = 1,450 M⁻¹ cm⁻¹. The specific activity of the fumarase used in this study was 550 units/mg.

Analysis of the results was performed using the program "Leonora," as described by Cornish-Bowden (25). With this program, kinetic measurements were fitted to the classical Michaelis-Menten equation by the method of least squares using dynamic weights (i.e. Leonora was allowed to select by itself the best weighting scheme from internal evidence in the experimental data obtained).

Spectroscopy—UV-irradiation spectra and enzyme activity measurements were determined with a double-beam Shimadzu 120 UV apparatus (Bausch and Lomb). Circular dichroism spectroscopy was performed on a Jasco 750 spectropolarimeter. The mean residue ellipticity [θ] was calculated from the equation [θ]u = (MRW/100) × (θu/λd), where the mean residual weight (MRW) is 110, c is the enzyme concentration in g/ml, and d is the path length in dm.

Determination of Dissociation Constants—Kd values for several substrates and inhibitors were determined from their inhibition against fumarase inactivation brought about either by urea or by chemical modification or proteolytic degradation. In all cases, first-order kinetic constants of inactivation are observed, and (log % activity) is plotted against time. As was described earlier by Beeckmans and Kanarek (15), the dissociation constant of an inhibitor I can then be calculated from: Kd = E/E1 × I, in which E/E1 is determined from the equations: k1 = E + GI = k and (E + I) = 1, which is based upon the fact that free enzyme, E, and fully occupied enzyme, EI, are inactivated at different rates, respectively, k1 and k2 is the rate constant at an intermediate inhibitor concentration I. The kinetic constants are calculated from: k1t0½ = 0.693, where t0½ is the half-life time of the inactivation.

When Kd is determined from circular dichroism data, the following equation is used: E/E1 = [θ]u - [θ]u(θ) - [θ]u - [θ]u(θ). The [θ]u and [θ]u are, respectively, the mean residue ellipticity, at a certain wavelength, of the free enzyme E and fully occupied enzyme EI, and [θ]u is the ellipticity at an intermediate concentration [I].

All these studies were performed in 10 mM Tris acetate buffer in order to avoid any influence of anions (like Cl⁻, K+ etc.) or substrate analogs, these events, together with the release of L-malate, will be rate-determining.

Recent crystallographic studies on the prokaryotic E. coli fumarase (class II) revealed the presence of two distinct binding sites per monomer for inhibitors (5–7). It was concluded from these studies that the active site (site A) comprises amino acid residues belonging to three different subunits. Residues Thr-100, Ser-139, Ser-140, and Asn-141 from the b-subunit, Thr-187, His-188 from the d-subunit, and Lys-324, Asn-326 from the c-subunit were supposed to form direct hydrogen bonds with substrates and inhibitors (5, 6). Moreover a highly coordinated buried water molecule was found at the A site and supposed to be associated to residues Ser-98, Thr-100, Asn-141 from the b-subunit and His-188 from the d-subunit (6, 7). An additional binding site (site B) is supposed to be nearby and is built up of residues belonging to one single subunit (the b-subunit), i.e. Arg-126, His-129, Asn-131, and Asp-132 (5). Conclusive evidence that site A is indeed the enzyme’s catalytic site was provided by site-directed mutagenesis experiments (7). Indeed, the mutation H188N in the A-site resulted in an enzyme showing largely reduced specific activity, whereas the mutation H129N in the B-site was essentially without effect.
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amount of a stock solution of benzylbromide (2 M reagent in methanol) to reach a final concentration of 20 mM (26). Since benzylbromide is only slightly soluble in water, each fumarase solution was continuously stirred during the modification reaction.

For studies of limited proteolysis, either trypsin at pH 8.0 or subtilisin at pH 7.3 were used. For trypsinolysis, a stock solution was made of 5 mg of trypsin (TPCK-treated) in 1 ml of 1 mM HCl. A ratio 1:5 (w/w) of trypsin:fumarase was used for digestion. For subtilisinolysis, a stock solution, consisting of 40 mM reagent in absolute ethanol. All solutions were buffered with 10 mM Tris acetate. Reaction velocities are expressed relative to $V_{\text{max}}$.

Eadie-Hofstee plots show that the calculated $K_m$ values were comparable with the affinity for the substrate of the proposed second substrate-binding sites; it is not taken into account the experimental points at low substrate concentrations. As can be seen from Table I, part A, values of $K_m$, $K_{i1}$, and $K_{i2}$ depend upon pH in a different way. The values for both $K_m$ and $K_{i1,2}$ are found to fulfill the Haldane relationship within experimental error: $K_{eq} = 4.4 = ([M/[F]]_{eq} = (V_mF \times K_{mF})/V_{max}(V_mF \times K_{p})$, where $K_m$ and $K_{p}$ are $K_m$, $K_{i1,2}$ or $K_{i2}$ for malate, respectively, fumarate, and $V_{max}$ the corresponding $V_{max}$ for malate, respectively, fumarate, determined in the same substrate concentration range.

Based on the results obtained, an estimation can be made, at different pH values, of the apparent values $K_{m,app}$ and $K_{i,app}$ for the equilibrium mixtures of L-malate and fumarate (Table I, part A) by using the equations (16), $K_{m,app} = 5.4/(1/K_{m,app} + (4.4/K_{m,app}))$ and $K_{i,app} = 5.4/(1/K_{i,app} + (4.4/K_{i,app}))$. From a number of ligands used in this study, their effect on fumarase kinetics was investigated. d-Malate ($K_m = 50$ mM at pH 7.3) and oxaloacetate ($K_m = 8.5$ mM at pH 7.3) are considered (16) to be structural analogues of, respectively, L-malate and fumarate ($K_m = 24$ mM) and fumarate ($K_m = 5.2$ mM). Also glycine is a competitive inhibitor of fumarase and we determined its inhibition constant to be $K_i = 4$ mM (results not shown; measurements performed in 10 mM Tris acetate buffer, pH 7.3, with L-malate concentrations below 1 mM). Moreover, several benzenecarboxylic acids were found to be competitive inhibitors. Their inhibition constants were determined in 10 mM Tris acetate buffer at pH 7.3 and are given in Table II. Especially pyromellitic acid, which can be considered to be a “duplicated” analog of fumarate, appears to be an excellent inhibitor and was used before as affinity ligand for the purification of fumarases from several organisms (12, 24, 28). Its $K_i$ of 0.6 mM (at pH 7.3) is 1–2 orders of magnitude lower than the $K_m$ for the natural substrates. However, the presence of an additional carboxyl group appreciably interferes with binding onto fumarase: the affinity of the enzyme for benzenepentacarboxylic acid is weaker by a factor 10 and becomes comparable with the affinity for the benzenetricarboxylic acids. The phthalate compounds, as well as the hexacarboxylic acid (mellitic acid), bind with less affinity to the enzyme. Among the phthalates, the 1,4-isomer has the poorest structural resemblance to the substrates and has also the highest $K_i$. Finally the affinity, if any, of benzoic acid was found to be negligibly small. On the other hand, l-aspartate is not a competitive inhibitor, despite its structural analogy with L-malate. This compound, however, influences the kinetics of fumarase in the same way as phosphate ions: simple Michaelis-Menten kinetics are observed in the presence of these compounds and one single $K_m$ ($K_i$) value can be determined (Table I, part B).

Protection by Ligands against Urea-induced Denaturation of Fumarase—Denaturation of fumarase by 2.5 M urea in 10 mM Tris acetate buffer, pH 7.3, follows first-order kinetics. The inactivation is markedly slowed down in the presence of substrates (the equilibrium mixture malate/fumarate), and by inhibitors (oxaloacetate and d-malate). As an example, protection by different concentrations of d-malate is shown in Fig. 2. Dissociation constants were calculated to be, respectively, $K_d$(L-malate/fumarate) = 250 [$\mu$M], $K_d$(oxaloacetate) = 130 [$\mu$M], and
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TABLE I

Kinetic constants for the fumarase catalyzed reaction at different values of pH

The following symbols are used. \( K_{m1M} \) and \( K_{m1F} \) are Michaelis constants for, respectively, L-malate and fumarate, determined at low substrate concentrations (i.e. up to 1 mM) using the program Leonora (25). \( K_{m2M} \) and \( K_{m2F} \) are Michaelis constants for, respectively, L-malate and fumarate at high substrate concentrations (i.e. above 5 mM), but without taking into account the experimental points at low substrate concentrations; they were determined using the same computer program. \( K_{d1M} \) and \( K_{d1F} \) are substrate (respectively, malate and fumarate) concentrations where the velocity of the reaction is half-way between the first and the second maximal initial velocities \( V_{max1M} \) and \( V_{max2M} \) are the apparent values for the equilibrium mixture of L-malate and fumarate (calculated as described under “Materials and Methods”). \( V_{max1F} \) and \( V_{max2F} \) are initial velocities, expressed relative to \( v = 100 \) in standard conditions (i.e. 50 mM L-malate + 50 mM potassium phosphate at pH 7.9); they were again determined using the Leonora program (25).

| pH     | \( K_{m1M} \) | \( K_{m1F} \) | \( K_{d1M} \) | \( K_{d1F} \) |
|--------|---------------|---------------|---------------|---------------|
| 6.5    | 12.9 ± 1.1    | 6.2 ± 0.3     | 10.7 ± 0.4    | 6.3 ± 1.5     |
| 6.8    | 14.2 ± 0.7    | 4.9 ± 0.5     | 10.5 ± 0.3    | 8.4 ± 1.0     |
| 7.0    | 16.3 ± 0.4    | 4.8 ± 0.5     | 11.3 ± 0.3    | 9.1 ± 0.9     |
| 7.3    | 24.2 ± 0.5    | 5.2 ± 0.3     | 14.4 ± 0.2    | 10.0 ± 0.5    |
| 7.5    | 39.2 ± 0.4    | 5.7 ± 0.3     | 18.8 ± 0.2    | 10.5 ± 0.5    |
| 8.0    | 79.6 ± 0.8    | 6.2 ± 0.4     | 24.9 ± 0.4    | 10.0 ± 0.8    |
| 8.5    | 160.0 ± 0.9   | 8.8 ± 0.6     | 38.3 ± 0.5    | 5.8 ± 0.8     |

A: in 10 mM Tris acetate buffer

B: influence of phosphate ions and l-aspartate

TABLE II

Denaturation of fumarase by 2.5 M urea at pH 7.3

| Ligand                        | Concentrationa | \( K_i \)b | \( K_d \) | \( t_{50} \) | additional ligandc |
|-------------------------------|----------------|------------|----------|----------|-------------------|
| None                          |                |            |          | 2.7      | Gly               |
| Classical inhibitors          |                |            |          |          |                   |
| Malate/fumarate equilibrium mixture | 1              | 14.4       | 250      | 22       | 44                |
| D-Malate                      | 1              | 50         | 410      | 15       | 28                |
| Oxaloacetate                  | 1              | 8.5        | 130      | 12       | 26                |
| Benzenecarboxylic acids       |                |            |          |          |                   |
| Benzoic acid                  | 1              | 5,000      |          | 3        |                   |
| Phthalic acid (1,2-dicarboxyl-) | 1              | 660        |          | 7        |                   |
| Iso phthalic acid (1,3-dicarboxyl-) | 1              | 580        |          | 9        |                   |
| Tere phthalic acid (1,4-dicarboxyl-) | 1              | 1,620    |          | 18       |                   |
| Benzenetricarboxylic acid (1,2,3-) | 1              | 150        |          | 58       | 31                |
| Benzenetricarboxylic acid (1,2,4-) | 1              | 80         |          | 58       | 27                |
| Benzenetricarboxylic acid (1,3,5-) | 1              | 70         |          | 45       | 21                |
| Pyromellitic acid             | 1              | 0.6        | 0.65     | 660      | 660               |
| i.e. benzenetetracarboxylic acid (1,2,4,5) | 0.05      | 175        |          | 180      | 35                |
| Benzenepenta carboxylic acid  | 1              | 56         |          | 70       | 71                |
| Mellitec acid                 | 1              | 950        |          | 10       |                   |

\( a \) Due to the high absorbance of the benzenecarboxylic acids, concentrations higher than 1 mM could not be tested with accuracy.

\( b \) \( K_i \) values determined from kinetic experiments, using l-malate concentrations up to 1 mM.

\( c \) \( K_d \) values calculated from protection against denaturation by 2.5 M urea.

\( d \) Concentrations of the additional ligand were: Gly (glycine) 100 mM, Asp (l-aspartate) 200 mM, or Gly/Asp (glycine) 100 mM + (l-aspartate) 200 mM.

\( K_{D-(L-malate)} = 410 \mu M \). These values lie between \( K_{m1} \) and \( K_{m2} \) (Table I). It can thus be concluded that saturating exclusively the active site (site A) is not sufficient in order to efficiently protect fumarase against denaturation by 2.5 M urea.

The protection by a number of other ligands was tested as well and the results are shown in Table II, where the half-life times of inactivation are calculated from the first-order kinetics obtained. Several interesting features can be deduced from the data presented. 1) Aromatic tri-, tetra-, and pentacarboxylic acids were found to slow down the inactivation by 2.5 M urea to various extents. Especially pyromellitic acid is a remarkably efficient protector against this type of denaturation. Moreover, its inhibition constant (\( K_i = 0.6 \mu M \)) corroborates the dissociation constant of this compound which was estimated from protection against denaturation (\( K_d = 0.65 \mu M \)) (Fig. 2, Table II). The benzenecarboxylic acids and benzenepenta carboxylic acid, which were shown above to be good competitive inhibitors, also protect the enzyme against denaturation in 2.5 M urea. The three phthalic acids and mellitec acid hardly protect fumarase, and neither does benzoic acid. 2) Neither the competitive inhibitor glycine (100 mM), nor L-aspartate (200 mM), protect fumarase considerably, and a mixture of both compounds is only slightly slowing down the inactivation rate (Table II). Both compounds, however, affect the protective capacity of other
inhibitors in different ways. Glycine increases the protective action of the (malate/fumarate) equilibrium mixture, and of the substrate analogs D-malate and oxaloacetate, whereas L-aspartate has no effect. Since the difference in half-life time of inactivation is quite considerable, we were able to determine the dissociation constant of glycine on the protection by 1 mM D-malate, i.e. no glycine (1 μM (Δ)), 5 mM (○), 10 mM (△), 50 mM (●), and 100 mM (□). B, protection of fumarase by various concentrations of pyromellitic acid, i.e. no ligand (●), 1 μM (Δ), 2 μM (●), 5 μM (○), 50 μM (▲), 200 μM (□), 500 μM (●), and 1 mM (○). C, effect of various concentrations of glycine on the protection by 1 mM D-malate, i.e. no glycine (○), 2 mM (●), 5 mM (○), 10 mM (△), 50 mM (△), 100 mM (●), and 200 mM (□). D, effect of various concentrations of L-aspartate on the protection by 200 μM pyromellitic acid, i.e. no aspartate (□), 5 mM (●), 10 mM (△), 20 mM (▲), 50 mM (○), 100 mM (●), 200 mM (●), and 400 mM (□).

Protection by Ligands against Methionyl Modification with Benzylbromide—Benzylbromide was shown by Rogers et al. (26) to inactivate fumarase due to alkylation of one methionine residue per monomer in pig fumarase (15). It was concluded that an equilibrium mixture (malate/fumarate) protects the enzyme against inactivation, and a K_d = 78 μM was calculated which is in good agreement with the K_m,l=app = 25 μM (Table I). Full protection against this type of modification is thus already achieved when the active site (site A) is saturated.

From protection with pyromellitic acid at various concentrations we calculated a K_d = 1.75 μM (Table V), which is in good agreement with the dissociation constant determined at the same pH from protection against denaturation in urea. The protective inhibitor glycine on the other hand protects the enzyme very well (Table V). For this ligand, a K_d = 5.36 mM was determined which again agrees very well with the value obtained from the denaturation experiments with urea. Finally, L-aspartate has no effect, which could be expected since this ligand is not a competitive inhibitor.

**Protection by Ligands against Tyrosyl Modification with Tetranitromethane**—Previously we studied the modification at pH 8.0 with tetranitromethane of one essential tyrosine residue per monomer in pig fumarase (15). It was concluded that an equilibrium mixture (malate/fumarate) protects the enzyme against tyrosylation, and a K_d = 4.2 mM was determined (Table III). This agreement with the dissociation constant determined at the same pH from protection against denaturation in urea. The dissociation constant was determined which again agrees very well with the value obtained from the denaturation experiments with urea. Finally, L-aspartate has no effect, which could be expected since this ligand is not a competitive inhibitor.

Significant protection against methionyl modification is only observed with concentrations of the malate/fumarate equilibrium mixture above the K_m value (Table V). The dissociation constant was determined to be K_d = 4.2 mM, which is in good agreement with the apparent K_m = 6.5 mM at pH 8.0. Also pyromellitic acid protects the enzyme against this type of inactivation (Table V). Neither glycine nor L-aspartate were found to have any effect on the inactivation rates.

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**Table III**

| pH | t_1/2 pyromellitic acid | K_d (μM) |
|----|-------------------------|----------|
| 7.3| 2.7                     | ∞        |
| 8.0| 4.5                     | 70       |
| 8.3| 7                       | 16       |

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*1 mM pyromellitic acid maximally protects fumarase against denaturation in 2.5M urea.*
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TABLE V
Protection by several ligands against chemical modification and limited proteolysis of fumarase

| Type of modification | Agent          | pH  | Ligand                        | Concentrationa | t1/2 | Kd  |
|----------------------|----------------|-----|-------------------------------|----------------|------|-----|
| Tyrosine             | Tetranitromethane | 8.0 | None                          | 4 mM           | 25   | 78 μM|
|                      |                |     | Malate/fumarate               | 5              |      | 1.75 μM|
|                      |                |     | Pyromellitic acid             | 0.5            | 26   | 15 mM|
|                      |                |     | Glycine                       | 200            | 86   | 5.38 mM|
|                      |                |     | l-Aspartate                   | 200            |      |     |
| Methionine           | Benzylobromide  | 6.8 | None                          | 11.5           |      |     |
|                      |                |     | Malate/fumarate               | 50             | 43   | 4.2 mM|
|                      |                |     | Pyromellitic acid             | 1              | 42   | 1.00 mM|
|                      |                |     | Glycine                       | 200            | 12   |     |
|                      |                |     | l-Aspartate                   | 200            |      | 14  |
| Proteolysis          | Trypsin        | 8.0 | None                          | 85             |      |     |
|                      |                |     | Malate/fumarate               | 50             | 2,400| 2.0 mM|
|                      |                |     | Oxaloacetate                  | 50             | 2,400| 0.3 mM|
|                      |                |     | l-Aspartate                   | 50             | 1,180| 3.8 mM|
|                      |                |     | Oxaloacetate                  | 50             | 1,120| 1.1 mM|

a Ligand concentration giving maximal protection.

Limited Proteolysis—Native fumarase is quite resistant to proteolysis by trypsin and subtilisin. Initially, proteolytic attack occurs in a limited area of the fumarase molecules and a 46-kDa fragment is generated in both cases (Fig. 3). During trypsinolysis, additional bands of lower molecular mass are also observed, but with subtilisin the degradation of the intermediate goes very fast. In both cases, the equilibrium substrate mixture (malate/fumarate), as well as oxaloacetate, slow down the degradation. Dissociation constants were determined (Table V). It can be concluded that significant protection is only observed at concentrations above the K1/2 value. Both the high affinity (active) site A, and the low affinity site B should thus be occupied in order to obtain full protection.

A partially proteolyzed fumarase sample was prepared by treating the enzyme (1 mg/ml in 10 mM Tris acetate buffer, pH 7.3) with 12.5 μg of subtilisin at 25 °C for 1 h. The sample was then extensively dialyzed in order to remove all small fragments generated. N-terminal sequence analysis of this sample, containing a mixture of intact (50 kDa) and partially degraded (46 kDa) subunits revealed one single amino acid at each step in the order 1ASQDSFRIEY10, which corresponds with the N-terminal sequence of pig heart fumarase (29).

Ligand-induced Conformational Changes—The influence of ligands (D-malate, oxaloacetate, pyromellitic acid, glycine, l-aspartate, and several combinations of them) on the secondary structure of fumarase was studied at pH 7.3 by circular dichroism (CD) measurements in the near-UV region. The spectrum in the absence of ligands is shown in Fig. 4A. It is built up of two tryptophanyl triplets. Maxima in triplet I are observed, respectively, at 278, 285, and 292 nm. Triplet II on the other hand shows maxima at 258.5 and 265.5 nm; because of the existence of triplet I, the third maximum is only a shoulder around 272.5 nm. Both tryptophanyl residues of pig heart fumarase thus appear to contribute to the CD spectrum in the near-UV region.

When we titrate fumarase with the substrate analog D-malate, we observe changes in the CD spectrum, as is shown in Fig. 4, B and C. At ligand concentrations gradually filling up the high affinity sites (site A), we only observe an increase in the CD values in triplet I (Fig. 4B). From the results shown, we were able to calculate a dissociation constant for D-malate of Kd = 35 μM, which corroborates the value of Kd = 50 μM for the same ligand, as well as the value of K1/2M = 24 μM for the analog l-malate, as determined from kinetic measurements (Table I). Upon adding more D-malate, we also fill up the low affinity binding sites (site B). We now observe an increase in the CD values in triplet II (Fig. 4C). Moreover, a small red-shift is clearly observed in the triplet peaks (indicated by broken arrows): in the presence of 100 mM D-malate, the triplet maxima occur, respectively, at 259.5 and 266 nm. In this region, we calculated a dissociation constant for D-malate of Kd = 15 mM, which again agrees very well with the kinetically determined value of K1/2M = 10 mM for l-malate (Table I).

We also investigated the effect of other ligands on the CD spectrum in the near-UV region (Fig. 4D). l-Aspartate (200 mM), and also phosphate (100 mM), obviously induce the same spectral changes in fumarase as do high concentrations of D-malate (200 mM). On the other hand, the inhibitor glycine (100 mM) induces the same spectral changes as 0.5 mM D-malate, which is sufficient to saturate the enzyme’s active sites but not the additional binding sites.

The CD spectra in the presence of pyromellitic acid are quite remarkable (Fig. 4E). Indeed, a concentration of 5.5 μM pyromellitic acid is enough to induce the same spectral changes as 200 mM D-malate. A mixture of 5.5 μM pyromellitic acid and either 100 mM glycine or 200 mM D-malate still results in the same fumarase CD spectrum. However, a mixture of 5.5 μM pyromellitic acid and 0.5 mM D-malate induces the same spectrum as 0.5 mM D-malate alone.
DISCUSSION

According to the results presented in this paper, the enzyme fumarase possesses not only four active sites (sites A), which have high affinity for their substrates ($K_d$ being in the micromolar range), but also additional binding sites (sites B), which bind the substrates with lower affinity ($K_d$ being in the millimolar range). All experiments were performed in Tris acetate buffers, since a number of anions, such as e.g. phosphate, are known to bind into additional binding sites as well, thereby modifying the enzyme’s properties (1, 11).

The nature of both types of binding sites (A and B) was unraveled by experiments of enzyme denaturation (urea), modification (tyrosyl and methionyl residues), and limited proteolysis on the one hand, and by circular dichroism measurements on the other hand. In all cases, the effect of several ligands was investigated and dissociation constants could easily be calculated and compared with kinetically determined values of $K_m$ ($K_i$) and $K_{1/2}$. The ligand pyromellitic acid was found to play a pivotal role in this study. All experiments described are consistent with a fumarase model schematically depicted in Fig. 5.

The active site (site A) harbors the tyrosyl residue whose modification with tetraniotomethane results in loss of activity. Indeed, full protection by the substrate equilibrium mixture malate/fumarate against this type of inactivation already occurs when the high affinity binding sites are occupied, a $K_d = 78 \mu M$ (pH 8.0) was determined. Into this site glycine also binds, which was shown to be a competitive inhibitor ($K_i = 4 \mu M$). Glycine was moreover shown to protect fumarase against inactivation with tetraniotomethane, and a $K_d = 5.36 \mu M$ could be determined.

A methionyl residue is apparently located in, or in the vicinity of the additional, low affinity binding site (site B). Indeed, full protection against modification with benzylbromide was only observed with malate/fumarate concentrations saturating both binding sites. A $K_d = 4.2 \mu M$ (pH 6.8) could be determined for the substrate equilibrium mixture, which corroborates the kinetically determined value of $K_{1/2,app} = 6.5 \mu M$.

L-Aspartate, which is a structural analog of L-malate but which is not a competitive inhibitor and, as such, does not bind into the active sites, readily binds into the low affinity sites. This could be concluded from CD spectroscopy in the near-UV region. Indeed, aspartate was found to induce the same spectral changes in fumarase as high concentrations of D-malate, which also saturates the low affinity binding sites. However, binding of aspartate in site B was shown not to be sufficient in order to protect the enzyme against methionyl modification by benzylbromide.

Full protection against limited proteolysis only occurs when both the high and the low affinity sites are occupied. With trypsin as well as with subtilisin, a peptide with a molecular
mass of about 4 kDa is initially cleaved off. We postulate that this degradation, which results in loss of activity, occurs at the C-terminal site. Indeed, the region 421-423 YDKAAKIAKTR (i.e., about 40 residues before the C terminus) contains several lysine residues. This stretch of amino acids forms part of helix α18 in *E. coli* fumarase and, according to x-ray crystallographic analyses (5), is protruding from the fumarase molecule. This region might thus indeed be more accessible for proteolytic enzymes. Moreover Tyr-421, which is conserved in all fumarases except the Arabidopsis thaliana enzyme, was supposed to be the tyrosyl residue modified with tetraniitromethane and being located at the entrance of the active site cleft (6).

According to the results presented, pyromellitic acid, i.e., benzene-(1,2,3,4,5)-tetracarboxylic acid, binds both into the high and the low affinity binding sites, and, moreover, one molecule of this ligand is able to bind into one site A and one site B at the same time, thereby (non-covalently) cross-linking the enzyme’s subunits; the affinity of fumarase for pyromellitic acid is very high (K_{d} is 0.6–2.2 μM, depending upon the pH). This scheme is purely schematical and does not intend to give a representation of the actual shape of the fumarase molecule. Moreover, residues involved in ligand binding in the high affinity sites (shaded area in this figure) are known in *E. coli* fumarase to belong to three different subunits.

Our results indicate that the competitive inhibitor glycine binds into the enzyme’s active sites. The affinity of fumarase for this ligand was determined in several ways: kinetic measurements lead to a value of K_{i} = 4 mM, pH 7.3; from protection studies against urea-induced denaturation we calculated a K_{d} = 3.7 mM (pH 7.3); finally, from protection against tyrosyl modification a K_{d} = 5.36 mM, pH 8.0, was observed. Nevertheless, this ligand clearly binds in a different way than the substrate analog D-malate. First, glycine protects fumarase appreciably more efficiently against tyrosyl modification by tetraniitromethane. Second, it is clear from CD spectroscopy that glycine does not prevent pyromellitic acid from binding in site B, whereas D-malate does. Indeed, a mixture of pyromellitic acid (5.5 μM) (i) with 100 mM glycine results in the same spectrum as pyromellitic acid alone; (ii) with 200 mM D-malate again gives the same spectrum, indicating that site B is still occupied; (iii) with 0.5 mM D-malate (which is only sufficient to saturate site A) results in the same spectrum as 0.5 mM D-malate alone, indicating that pyromellitic acid does not bind anymore in site B.

Finally, we could wonder what might be the advantage and/or the reason for fumarase to have additional, low affinity substrate-binding sites. This enzyme already shows high catalytic efficiency, as measured from the values of k_{cat}/K_{m}, which, at pH 7.3, amount, respectively, to 1.54 × 10^{8} M^{-1} s^{-1} in the direction of fumarate consumption (K_{cat}/K_{m} = 5.2 × 10^{-6} M and k_{cat} = 800 s^{-1}), and to 3.75 × 10^{7} M^{-1} s^{-1} in the direction of fumarate formation (K_{cat}/K_{m} = 24 × 10^{-6} M and k_{cat} = 900 s^{-1}). The increase in activity upon filling up the low affinity binding sites was found to be only moderate (maximally 2-fold), so that...
it is doubtful that reaching higher catalytic rates might have been the ultimate goal for the existence of sites B. What might be the significance of the second binding site still remains purely speculative. Since indications accumulate pointing to the existence of physical interactions between enzymes of the same metabolic pathway (31–33), one might speculate, e.g., on local conformational changes within the enzyme, resulting in stronger interactions with the neighboring enzymes. The influence of phosphate on fumarase may in that case be considered from the same point of view. The citric acid cycle complex will indeed be localized in the immediate vicinity of mitochondrial ATPase, where the local concentrations of phosphate might be relatively high. Moreover, 100 mM phosphate induces about the same CD spectrum in the near-UV region as 100 mM d-malate. Another clue toward the reason for the existence of the sites B could perhaps be found in the ability of aspartate to fit into these sites. Fumarase and aspartase are known to show extensive sequence homology (34), and also the latter enzyme seems to possess additional substrate-binding sites (35–38). It does not seem unlikely then that both enzymes would have evolved from an ancestral gene through duplication, leading to the creation of two separate binding sites. A binding site on fumarase for aspartate could then be a reminiscence of the common origin of both enzymes. Although we could demonstrate recently that, indeed, regions of internal sequence homology are found both in fumarase and aspartase, the location of these homologous stretches within both enzyme molecules seems to rule out such a simplistic view upon their evolution.

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