The Subunit Interactions of Fumarase*

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SUMMARY

The subunit interactions of fumarase have been examined as a function of enzyme concentration, pH, and concentration of guanidine hydrochloride. Fumarase was found to dissociate, with loss of activity, either at low concentrations of the enzyme ( <1 × 10^{-4} mg per ml), below pH 6, above pH 10, or in solutions of guanidine hydrochloride ( >1 M). At pH 9 a species of enzyme was produced which was fully associated, but whose hydrodynamic structure was different from that of the native enzyme.

Fumarase dissociated in dilute acid or alkali or by guanidine hydrochloride could be reassociated with nearly complete recovery of activity. The reassociation of the acid-dissociated fumarase was observed to follow first order kinetics with respect to enzyme concentration. L-Malate, citrate, phosphate, and ATP all reduced the rate of dissociation and increased the extent of recombination of the subunits.

As judged by optical rotatory dispersion measurements, little change in polypeptide chain conformation was noted between native fumarase and the enzyme dissociated between pH 5 and 11. The third groups of the dissociated enzyme, however, were found to be more exposed to solvent than the same groups located in the tetrameric molecule.

Previous studies have shown that fumarase (fumarate hydratase, EC 4.2.1.2) from swine heart muscle has a molecular weight of 194,000 and is composed of four identical polypeptide chain subunits (1). These subunits are held together by non-covalent interactions since the enzyme may be dissociated into 48,500 molecular weight species in the presence of 6 M guanidine hydrochloride (1). Fumarase was also dissociated by urea at concentrations >0.25 M or by modification of its thiol groups (2). In both cases dissociation was accompanied by loss of enzymic activity.

Dimeric species possessing some residual structure. In both cases dissociation was accompanied by loss of enzymic activity.

In the studies reported here, the subunit structure of fumarase was examined as a function of enzyme concentration, pH, and guanidine hydrochloride concentration, three variables which normally affect subunit interactions. The gross conformational features and catalytic properties of the subunit species produced give additional insight into the structure-function relationships of fumarase.

EXPERIMENTAL PROCEDURE

Fumarase—Fumarase was prepared from swine heart muscle by the method of Kanarek and Hill (3), and the crystalline enzyme was solubilized in 0.01 M potassium phosphate, pH 7.3, as described earlier (4). Solutions of fumarase in other buffers of the desired pH were prepared by dialyzing the enzyme against at least a 2000-fold excess of the appropriate buffer for 4 hours at room temperature. The thin film dialysis technique of Craig (5) was used to hasten equilibration.

The specific activity of fumarase was normally determined spectrophotometrically as described earlier (3). Solutions of the enzyme which had been partially dissociated below pH 6 or above pH 8 were assayed at 5°C in 0.05 M L-malate-0.05 M phosphate at pH 7.3. Under these conditions, rapid reactivation of the enzyme, observed during the course of the assay at room temperature, was not encountered. A conversion factor of 4.92 (ratio of the activity of native fumarase at 25°C to its activity at 5°C) was used to correct the observed activity to 25°C. The concentration of fumarase solutions was measured at 250 nm, where a 1 mg per ml solution of the enzyme has an absorbance of 0.51 (3).

Analytical Methods—Spectral measurements were made on a Zeiss PMQ II spectrophotometer. The pH of all solutions was determined in the following manner. Fumarase (2.54 mg per ml) was inactivated by exhaustive dialysis against 0.01 M potassium phosphate, pH 5.0, and at protein concentrations >1 mg per ml, runs were performed at 50,780 rpm with 12-mm cells and at protein concentrations <1 mg per ml at 50,740 rpm in a 30-mm cell. Sedimentation velocity measurements of fumarase in guanidine hydrochloride at concentrations >0.25 M were made at 42,040 rpm with a synthetic boundary cell.

Reactivation—The rate of reactivation of acid-dissociated fumarase, measured as a function of enzyme concentration, was determined in the following manner. Fumarase (2.54 mg per ml) was inactivated by exhaustive dialysis against 0.01 M potassium acetate at pH 5.0. Aliquots (5, 50, and 250 μl) of the partially dissociated enzyme were then added to 3 ml of 0.5 M L-malate-0.5 M phosphate, pH 7.4 at 25°C. Reactivation was monitored by measuring the increase in the rate of conver-
sion of L-malate to fumarate spectrophotometrically at 250 nm. When this assay exceeded the range of zero order kinetics, aliquots of the enzyme were removed with time and assayed in a second substrate solution containing 0.05 M L-malate and 0.05 M phosphate at pH 7.3. The percentage reactivation was determined from the fumarase activity measured at any one time relative to the activity of the fully reactivated enzyme.

Titration with p-Mercuribenzoate—Solutions of p-mercuribenzoate at pH 7 were prepared as reported earlier (4). The concentration of these solutions was determined spectrophotometrically as described by Boyer (6).

The rate of reaction of the thiol groups of fumarase with mercuribenzoate as a function of enzyme concentration, was determined in the following manner. Solutions of fumarase (1.18 and 0.09 mg per ml) in 0.01 M Tris-acetate at pH 7.0 were incubated in a thermostated vessel at 25°C. The reaction was initiated by the addition of an equal volume of 4.8 × 10⁻³ m mercuribenzoate, pH 7.0, at 25°C. Aliquots were withdrawn with time and assayed for fumarase activity. The extent of the reaction of the thiol residues was assumed to parallel the loss in enzymic activity (2, 4).

In order to measure the reaction of fumarase with mercuribenzoate as a function of pH, solutions of fumarase (1.2 mg per ml) in 0.01 M potassium acetate at pH 5.0, 5.5, or 7.0 were prepared by exhaustive dialysis against the appropriate buffer. To 0.75 ml of these solutions at 25°C, 0.25 ml of 3.5 × 10⁻⁴ M mercuribenzoate, pH 7.0, was added. The rate of reaction of the thiol groups was monitored spectrophotometrically at 290 nm as described earlier (4). The percentage modification of the thiol groups was determined from the change in absorbance at any one time relative to the total change in absorbance when the reaction was complete.

Reagents—p-Chloromercuribenzoate and ATP were purchased from Sigma. L-Malate (A grade) was obtained from Calbiochem and citric acid from J. T. Baker Chemical Company. All were used without further purification.

Guanidine hydrochloride (J. T. Baker Chemical Company) was recrystallized from ethanol (7) and stored over silica gel.

Sephadex G-200 (Pharmacia) was prepared for use by swelling the gel in water at 80°C overnight, followed by equilibration in the appropriate buffer.

**EXPERIMENTAL RESULTS**

**Effect of Enzyme Concentration on Dissociation of Fumarase**

**Degree of Dissociation**—At an enzyme concentration of 0.03 mg per ml, fumarase in dilute phosphate buffer at pH 7.3 was shown by sedimentation-equilibrium analysis to be fully associated and composed of four subunit polypeptide chains (1). In order to estimate whether this tetrameric species was the catalytically active form of fumarase under normal assay conditions, where its concentration is from 5 × 10⁻⁴ to 5 × 10⁻⁵ mg per ml, the chromatographic behavior of very dilute solutions of fumarase on Sephadex was compared with the behavior of the enzyme at higher concentrations where it is known to be fully associated. The results of this experiment, which are shown in Fig. 1, indicated that the enzyme eluted in essentially the same volume irrespective of its concentration.

All of the enzymic activity applied to the column was recovered in the column eluates, thus the activity profiles shown in Fig. 1 must also represent the protein concentration profiles. Since the enzyme concentrations in the peak fractions were approximately 10 times more dilute than the samples originally applied to the column, this indicates that at a protein concentration approaching 1 × 10⁻⁴ mg per ml neither inactivation nor appreciable dissociation of fumarase had occurred. A completely dimeric species would be expected to have emerged at 175 ml, or considerably later than the peaks observed in Fig. 1.

**Stability of Inactivation**—In contrast to the stability of low concentrations of fumarase in phosphate buffer, equivalent amounts of the enzyme in potassium acetate spontaneously lost activity. The rate of inactivation of fumarase in dilute acetate solutions at pH 1.3 and 5.0, as a function of enzyme concentration, is shown in Fig. 2.

Several aspects of these studies are noteworthy. First, it is apparent that, at both pH values, the rate of inactivation of fumarase increased as the initial concentration of the protein was decreased. The same phenomenon was observed when the above experiments were performed in silicone-coated glassware.

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**Fig. 1.** Chromatography of dilute and concentrated solutions of fumarase on Sephadex G-200. Solutions of fumarase at initial concentrations of 3.14 mg per ml and 1 × 10⁻⁴ mg per ml in 5 × 10⁻³ M phosphate, 1 × 10⁻³ M 2-mercaptoethanol, pH 7.3, were chromatographed on a column (2 × 74 cm) of Sephadex G-200 equilibrated with the same buffer. The column was run at room temperature at a flow rate of 12 ml per hour. Effluent fractions (1 ml) were monitored for enzymic activity in 0.05 M L-malate-0.05 M phosphate. The void volume of the column was 85 ml.

**Fig. 2.** Effect of enzyme concentration on the rate of inactivation of fumarase at pH 7.3 and 5.0. The rate of inactivation of fumarase at the enzyme concentrations indicated was measured in 0.01 M potassium acetate at pH 7.3 (A) and pH 5.0 (B) at 25°C.
which reduces adsorption and thus denaturation of the enzyme on the surface of the reaction vessel. Secondly, inactivation appeared to be at least a two-step reaction as judged from the break in the slopes of the first order rate curves. Thirdly, the enzyme was considerably more labile at pH 5.0 than at pH 7.3. Finally, the inactivation observed at low concentrations of fumarase appeared to be irreversible. Attempts to reactivate the denatured enzyme by incubation in 0.05 M L-malate-0.05 M phosphate at pH 7.3, conditions which stabilize equivalent concentrations of the native enzyme, were unsuccessful.

Low concentrations of L-malate and the competitive inhibitors, citrate, phosphate, and ATP decreased the rate of inactivation of fumarase. The stabilizing effects of citrate and ATP on the spontaneous denaturation of the enzyme in potassium acetate are shown in Fig. 3.

Reactivity of Thiol Groups—Fumarase possesses 12 thiol groups which appear to be located in a hydrophobic region of the molecule (4). The rate of reaction of these groups with p-mercuribenzoate has been studied as a function of enzyme concentration as shown in Fig. 4. Clearly, the thiol groups reacted faster at the lower concentration of fumarase. This difference in reactivity cannot be attributed to significant differences in the free concentration of mercurial since sufficient excess of the reagent was used to insure its concentration remained relatively constant during the course of reaction with both high and low concentrations of fumarase.

Effect of pH on Dissociation of Fumarase

Sedimentation Velocity—The activity and sedimentation-velocity behavior of fumarase in Tris-acetate between pH 4.5 and 11.3 are shown in Fig. 5. A slower moving component, which first appeared at pH 6.0, was observed in increasingly larger amounts as the pH was lowered. At pH 4.5, a bimodal boundary was still observed although the faster sedimenting component became heterogeneous. In the alkaline region a single boundary was observed until pH 10, where a minor component first appeared. There was an increase in concentration of this component as the pH was increased, until at pH 11.3, it became the only detectable species. It is evident that the changes in sedimentation coefficients of the tetrameric and dimeric species, as observed in the acidic and alkaline regions, are accompanied by gross inactivation of the enzyme. Between pH 5.0 and 6.0, the percentage loss in activity closely paralleled the weight fraction of the slower sedimenting component. The relationship between subunit structure and activity in the alkaline region was less apparent.

The distribution between the slower and faster sedimenting species observed in the acidic pH region does not represent an equilibrium mixture of these components, since the concentration of the slower moving species gradually increased with time. This increase was accompanied by a proportionate decrease in the specific activity of the enzyme. Although the relative concentrations of the two components varied with time, the sedimentation coefficients of each species remained unchanged over the interval investigated. Furthermore, it was found that the relative concentrations of the faster and slower sedimenting species at a concentration of 0.5 mg per ml of fumarase were essentially the same as those observed at a concentration of 6 mg per ml.

Fig. 6 shows the concentration dependence of the sedimentation coefficient of fumarase, at pH 7.3 and 9.0. The sedimentation coefficients at pH 9.0 are lower than those measured for the native enzyme at pH 7.3.
Since, theoretically, macromolecules are fractionated on Sephadex at pH 9.0 the activity peak eluted slightly after the protein peak. Curves of fumarase are shown in Fig. 8. These data suggest Sephadex G-200 revealed that at pH 5.5 the peak containing enzymic activity eluted slightly before the protein peak, whereas at pH 9.0 the enzymically active component of fumarase is larger according to their size, the above observations suggest that at pH 5.5 the enzymatically active component of fumarase has a molecular weight of 5.84 X 10^13 set by assuming that the sedimentation coefficient of the enzyme varied as the two-thirds power of the sedimentation coefficient of the enzyme at pH 7.3. The value of s and g was estimated to be 5.84 X 10^-13 sec by assuming that the sedimentation coefficient of the enzyme varied as the two-thirds power of the molecular weight. Inspection of Fig. 6 reveals that the dependence on protein concentration for fumarase at pH 9.0 does not conform with the theoretical curve indicating that the enzyme is not appreciably dissociated at this pH. Essentially the same results were also observed at an ionic strength of 0.1 M NaCl, suggesting that the decrease in s and g at pH 9.0 is not a result of electrostatic interactions.

The sedimentation velocity patterns and the activity of fumarase in citrate-phosphate buffer were also determined at several pH values. The results of these studies are presented in Fig. 7. In contrast to the behavior of fumarase in Tris-acetate at pH 5.0, the enzyme in the citrate-phosphate buffer was apparently unaltered structurally or catalytically. The stabilizing effects of the citrate and phosphate anions were less pronounced in the alkaline region. Although fumarase at pH 9.0, in the presence of these anions, was nearly fully active and structurally intact, the enzyme at higher pH values was readily denatured.

Sephadex Chromatography—Chromatography of fumarase on Sephadex G-200 revealed that at pH 5.5 the peak containing enzymic activity eluted slightly before the protein peak, whereas at pH 9.0 the activity peak eluted slightly after the protein peak. Since, theoretically, macromolecules are fractionated on Sephadex according to their size, the above observations suggest that at pH 5.5 the enzymatically active component of fumarase is larger than the inactive component, while at pH 9.0 the opposite is true.

Optical Rotatory Dispersion—The optical rotatory dispersion curves of fumarase are shown in Fig. 8. These data suggest that changes in pH between 5.0 and 11.0, which profoundly affected the subunit structure and catalytic activity of fumarase, did not appreciably alter the polypeptide chain conformation of the enzyme.

Reactivity of Thiol Groups—The rate of reaction of the thiol groups of fumarase with p-mercuribenzoate at pH 5.0, 5.5, and 7.0 is shown in Fig. 10. The percentage of enzyme in the dis-
The rate of reaction of the thiol groups with p-mercuribenzoate was measured in 0.01 M potassium acetate at pH 5.0 (○), pH 5.5 (△), and pH 7.0 (☆) at 25°. The rate of reaction of the thiol groups was monitored spectrophotometrically as described under “Experimental Procedure.” The dashed lines refer to the percentage of fumarase in the dissociated state at these same pH values, as determined by specific activity measurements, at the pH values indicated, relative to the specific activity of the native enzyme (33,000 units per mg).

**FIG. 10.** Reactivity of the thiol groups of fumarase as a function of pH. The rate of reaction of the thiol groups of fumarase with p-mercuribenzoate was measured in 0.01 M potassium acetate at pH 5.0 (○), pH 5.5 (△), and pH 7.0 (☆) at 25°. The rate of reaction of the thiol groups was monitored spectrophotometrically as described under “Experimental Procedure.” The dashed lines refer to the percentage of fumarase in the dissociated state at these same pH values, as determined from the specific activity of the enzyme, at the pH values indicated, relative to the specific activity of the native enzyme (33,000 units per mg).

**FIG. 11 (left).** Denaturation of fumarase at several concentrations of guanidine hydrochloride as a function of time. The change in $s_{20,w}$ of fumarase (∼0.5 mg per ml) in 0.01 M phosphate, 0.05 M mercaptoethanol at pH 7.3, containing the concentrations of guanidine hydrochloride indicated, is shown as a function of time (top). The rate of inactivation of the enzyme under the same conditions is presented (bottom). Denaturation was measured at room temperature.

**FIG. 12 (right).** Decrease in the activity and $s_{20,w}$ value of fumarase as a function of guanidine hydrochloride concentration. The specific activity and $s_{20,w}$ values of fumarase (∼0.5 mg per ml) in 0.01 M phosphate, 0.05 M mercaptoethanol, pH 7.4 were measured as a function of increasing guanidine hydrochloride concentration. These values were measured after 10 hours incubation in the denaturant at room temperature.

**Discussion**

An investigation of fumarase at low protein concentration revealed that the stability of the enzyme to inactivation was markedly dependent upon the presence of certain anions. Low concentrations of fumarase (∼1 × 10^{-4} mg per ml) in Tris-acetate were observed to lose activity spontaneously, whereas equivalent concentrations of the enzyme in L-malate, citrate, ATP, or phosphate remained active for much longer intervals. Although the exact mechanism by which these compounds protect fumarase is unknown, several observations suggest that the stability of the enzyme is related to its degree of association. (a) An investigation of the denaturation of fumarase in Tris-acetate, as a function of enzyme concentration, revealed that as the concentration of protein was lowered, its rate of inactivation increased. Since dilution of a subunit protein favors its dissociation, the above observation indicates that dissociation of fumarase in Tris-acetate is responsible for inactivation of the enzyme. (b) Equally dilute concentrations of fumarase, stabilized in the presence of 0.05 M potassium phosphate, remained appreciably associated, as judged by molecular sieve chromatography. (c) Phosphate and citrate stabilized the tetrameric structure of fumarase at acidic pH values, whereas, in the absence of these buffers, the enzyme was extensively denatured.

**Table I**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Recovery of activity</th>
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<tr>
<td>0.05 M 2-mercaptoethanol, pH 7.0</td>
<td>16.5</td>
</tr>
<tr>
<td>0.05 M phosphate-0.05 M 2-mercaptoethanol, pH 7.3</td>
<td>84</td>
</tr>
<tr>
<td>0.05 M L-malate-0.05 M phosphate-0.05 M 2-mercaptoethanol, pH 7.3</td>
<td>95</td>
</tr>
</tbody>
</table>
As discussed below, this denaturation appears to be related to dissociation of the enzyme.

The finding that L-malate and the competitive inhibitors, phosphate (9), citrate (10), and ATP (11), stabilize the tetrameric structure of fumarase implies that these compounds, in addition to interacting with the active site of the enzyme, also influence subunit interactions remote from the active site. In light of this observation, the finding that substrates and competitive inhibitors often protect an enzyme against inactivation by "affinity labels" cannot be taken as unequivocal evidence that these reagents are reacting with structures in the active site. In the case of fumarase, for example, substrate protection would be observed if the modification of residues distant from the active site, but more accessible in the dissociated form of the enzyme, resulted in inactivation. This type of protection, indeed, appears to explain the decreased reactivity of the thiol residues of fumarase in the presence of L-malate or phosphate (2, 4).

Sedimentation velocity analysis of fumarase in Tris-acetate between pH 4.5 and 11.3 revealed significant structural alterations in the enzyme. The \( s_{20,w} \) values of the fumarase species produced in both the acidic and alkaline regions were considerably lower than that determined for the active enzyme. Unfortunately, it was not possible to determine the molecular weights of these components by sedimentation equilibrium analysis, since solutions of the enzyme at pH values <6.5 and >8.0 precipitated before equilibrium could be established. The gross structural features of some of the subunit species produced, however, could be estimated with the aid of other physical measurements.

The optical rotary dispersion curves of fumarase were essentially unchanged within the pH range 5.0 to 11.0. This observation implies that the slower sedimenting species detected by ultracentrifugal analysis, must result principally from alterations within the subunit (or quaternary) structure of the enzyme, rather than from conformational changes within the polypeptide chain (or tertiary) structure of the enzyme. Two types of changes in subunit interactions may be envisioned which might give rise to a decrease in the sedimentation coefficient of fumarase. The enzyme could dissociate to yield a lower molecular weight species, or the hydrodynamic structure of the fully associated enzyme could be altered such that the frictional coefficient of the protein was increased.

Sedimentation velocity measurements of fumarase in Tris-acetate between pH 6.0 and 4.5 revealed two components, a faster sedimenting species resembling native tetramer and a slower moving component which increased in concentration as the pH was lowered. Since the weight percentage of the slower component directly paralleled the percentage inactivation of the enzyme, this species was judged inactive. These observations, together with the finding that the inactive component eluted later on Sephadex than the active enzyme, lead to the conclusion that the slower sedimenting species is smaller than the native protein, and therefore represents a dissociation product of the tetramer.

Fumarase in Tris-acetate at pH 9.0 sedimented as a single boundary with an \( s_{20,w} \) of 7.8, approximately 1 S lower than the native enzyme at the same protein concentration. Two observations suggest that this decrease in \( s_{20,w} \) results from an increase in the frictional coefficient of the enzyme. Firstly, the decrease in the sedimentation coefficient does not appear to result from a decrease in the molecular weight of the enzyme, as judged by the dependence of the \( s_{20,w} \) value of the pH 9.0 component on protein concentration. As outlined under "Experimental Results," if the boundary observed at pH 9.0 represents a rapid equilibrium between tetramer and a lower molecular weight species, then the observed sedimentation coefficients should decrease as the concentration of protein is lowered. It was found, however (Fig. 6) that the \( s_{20,w} \) of the boundary varied with total enzyme concentration in a manner similar to that expected for a fully associated protein. Secondly, when fumarase, at pH 9.0, was chromatographed on Sephadex, the fraction possessing enzymic activity eluted after the protein peak. Since all previous observations indicate that the associated, or tetrameric, form of fumarase is the active species of the enzyme, the above result suggests that the inactive fraction either possesses a greater hydrodynamic volume or is more asymmetrical than the native enzyme. Increased hydration of the enzyme at pH 9.0 could account for a larger hydrodynamic volume, whereas an increase in asymmetry could result from a less compact arrangement of the individual subunits within the tetrameric species. Although it is not known which mechanism prevails both structural alterations will lead to increased frictional coefficients and hence lower \( s_{20,w} \) values. Positive preferential hydration of the enzyme could also partially explain the observed decrease in \( s_{20,w} \), however, at the low concentrations of buffer used, this effect should be negligibly small relative to that of the change in frictional coefficient.

The structural properties of the slower sedimenting component, first observed at pH 10.0, were not examined, however, the low \( s_{20,w} \) range of the species (4.8 to 2.1) relative to that observed for the native enzyme \( (s_{20,w} = 8.8) \) strongly suggests that this component represents a dissociation product of the tetramer.

The reactivation of acid-denatured fumarase was found to be a first order reaction. This observation suggests that either (a) the rate-limiting step of reactivation represents small changes within the polypeptide chain structure of the dissociated subunits followed by rapid association to the active tetramer or (b) that conformationally unaltered subunits rapidly associate to form an inactive tetrameric molecule, followed by slow structural changes within this associated species to produce the native enzyme. It is interesting to note that the renaturation of fumarase dissociated in urea is a second order reaction (2), and thus that the reactivation of the urea-denatured enzyme probably proceeds by a different mechanism than that observed for the acid-denatured enzyme.

An investigation of the reactivity of the thiol groups of fumarase, as a function of enzyme concentration, revealed that a decrease in the concentration of the protein produced an increase in the rate of reaction of these cysteinyl residues with p-mercuribenzoate. Since the only property of fumarase which, theoretically, should be affected by dilution is its degree of association, the above observation suggests that the thiol groups of the dissociated subunits are more reactive than the same residues found in the tetrameric molecule. This view is in accord with the finding that the cysteinyl residues of the dissociated subunit species, produced at pH 5.0, were also more reactive than the thiol groups of the tetrameric component. The reaction of the residues of the dissociated species was, in fact, complete within seconds, suggesting that these groups are fully exposed to the solvent medium.

Earlier studies showed that the thiol groups of tetrameric fumarase are buried in a hydrophobic region of the enzyme (4). The evidence presented above, indicates that the thiol residues of the dissociated species are located at or near the surface of the
enzyme. If the conformation of the individual subunits is unaltered by dissociation or if small conformational changes accompanying dissociation do not appreciably affect the polypeptide chain structure in the vicinity of the thiol residues, then the change in the thiol environment observed upon dissociation implies that the cysteinyl residues are located at the subunits interfaces within the tetrameric molecule.

An examination of the denaturation of fumarase, as a function of guanidine hydrochloride concentration, revealed significant decreases in both the sedimentation rates and activity of the enzyme in the presence of low concentrations (0.5 to 1.0 M) of the denaturant. In contrast to the lability of fumarase in guanidine hydrochloride, previous studies have shown that the enzyme in urea was fully active in 1.5 M solutions of this reagent and dissociated only at urea concentrations of 2 M (2). These observations are in accord with the general understanding that guanidine hydrochloride disrupts hydrophobic and hydrophilic bonds much more effectively than urea.

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