The Effect of pH and Other Variables on the Dissociation of Beef Liver Glutamic Dehydrogenase*

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Crystalline beef liver glutamic dehydrogenase has been shown to exhibit a marked dependence of the sedimentation coefficient on protein concentration. Since this original observation by Olson and Anfinsen (1), it has been shown that the enzyme undergoes a reversible association-dissociation reaction in which enzyme of molecular weight 1,000,000 can dissociate into units of molecular weight of approximately 250,000 (2, 3). Aside from being dependent upon enzyme concentration, the extent of this reversible dissociation is affected by a number of reagents including 1,10-phenanthroline (2), phenanthridine (4), the coenzymes for the reaction, either in the presence or absence of various nucleotides (5), and steroids in the presence of coenzyme (6). Most of these effects have been observed in the course of determining how particular reagents would affect the activity of the enzyme, since it has been shown that reagents which enhance dissociation of this type also cause decreased enzymatic activity (2, 4-6). The purpose of the present paper is to report more systematic studies on the properties of the enzyme with respect to the factors which cause dissociation of the molecule.

It is shown that a large number of factors including pH, bicarbonate, urea, sodium dodecyl sulfate, and temperature may serve to disrupt the bonds that hold enzyme subunits together, but that there are two distinct types of dissociation. Thus the subunits of approximately 250,000 (2, 3). Aside from being dependent upon enzyme concentration, the extent of this reversible dissociation is affected by a number of reagents including 1,10-phenanthroline (2), phenanthridine (4), the coenzymes for the reaction, either in the presence or absence of various nucleotides (5), and steroids in the presence of coenzyme (6). Most of these effects have been observed in the course of determining how particular reagents would affect the activity of the enzyme, since it has been shown that reagents which enhance dissociation of this type also cause decreased enzymatic activity (2, 4-6). The purpose of the present paper is to report more systematic studies on the properties of the enzyme with respect to the factors which cause dissociation of the molecule.

EXPERIMENTAL PROCEDURE

Crystalline enzyme was obtained from C. F. Boehringer and Son, Germany. The enzyme was used without further recrystallization. In most cases, the crystals were obtained under ammonium sulfate, and no marked differences were observed between crystals suspended in ammonium sulfate and in sodium sulfate. Nucleotides were obtained either from Sigma Chemical Company or Pabst Laboratories. All other reagents were obtained commercially and used without further purification.

The sedimentation experiments were performed on a Spinco model E analytical ultracentrifuge.1 In almost all cases, two single-sector cells were used at a speed of 42,040 r.p.m. The temperature of most runs was 6-8° and was determined by measuring the temperature of the rotor before the sedimentation run. At a speed of 42,040 r.p.m., the temperature correction for cooling due to adiabatic expansion is small (less than 0.1°) and was neglected. The pH was measured before and after the sedimentation runs if the pH of the buffer solution was not near the pK value for that buffer. In most cases, the pH values did not change during the run. Also, in most cases, the pH value given is that of the solution at the temperature of the sedimentation run and not at room temperature. The viscosities of Tris-acetate solutions at various pH values were measured at 20° in an Ostwald type viscometer.

The light scattering experiments were performed with the Phoenix-Brice light scattering apparatus and a thermostated cell compartment for a cell which is 1 cm square (inside diameter) and contains approximately 3 ml of solution. The cell compartment consists of two brass blocks, one placed 2 cm above the other, attached by means of a right angle metal plate. In the side of the metal plate which faces the light source, a slit is cut for the incident light beam. The other side of the metal plate faces the rear of the instrument.

Water was circulated through the top brass block and through the platform on which the cell holder is seated. The cell was inserted through the top block and held in position by a hole in the bottom block. The metal plate is in close contact with two sides of the cell and is maintained at the proper temperature by conduction from the top and bottom blocks. The whole cell holder is painted a dull black to prevent secondary reflections. Temperature equilibration of the solution was attained within a few minutes. It is interesting to note that the scattering of the buffer solution did not change over a wide temperature range even though there were undoubtedly some convection gradients in the cell. Temperatures were measured in the cell with a small probe that could be inserted into the solution.

The activity of the enzyme was measured as described previously in 0.01 M Tris-acetate buffer at pH 8, 10^{-4} M EDTA, with 1 \times 10^{-4} M DPNH, 5 \times 10^{-2} M α-ketoglutarate, and 0.1 M NH₄Cl (5).

RESULTS

Sedimentation Coefficient as Function of pH—Fig. 1 shows the variation in s_{20,w} as a function of pH in Tris-acetate and phosphate buffers. The enzyme concentration in all experiments was 3.2 mg per ml, high enough so that most of the enzyme existed, at neutral pH values, in the associated form of molecular weight 1,000,000. The concentration of acetate ion at the diff-

1 The author would like to thank Miss Carmelita Lowry for performing the sedimentation velocity experiments described in this paper.
different pH values was maintained at a value of approximately 0.1 M and the pH adjusted with Tris. Above pH 9, Tris-acetate plus sodium acetate was used. As can be seen from Fig. 1, $s_{20,w}$ is essentially a constant from pH 6 to 10. In addition, provided that the protein concentrations are maintained constant, the sedimentation patterns are almost identical throughout this pH region. Below pH 6, $s_{20,w}$ falls to 12 S at pH 4.3. The solid line in Fig. 1 was calculated assuming that the minimal sedimentation coefficient was 12 S and that the points would fit a simple titration curve. From the experimental points one may determine the pK of this plot to be 4.8. The addition of 0.01 M 1,10-phenanthroline, which causes complete dissociation at neutral pH values (2), to enzyme at pH 4.3 causes no further dissociation. It is worth noting that at all pH values between 4 and 6, only a single peak is observed in the ultracentrifuge. Light scattering experiments at pH 4.2 and 6 show quite conclusively that the molecular weight is decreased by 4-fold at the lower pH. Furthermore, the light scattering experiments show that the dissociation is completed as rapidly as measurements can be made, less than 1 minute after adjusting the pH. Although in the range pH 4.2 to 5, the enzyme becomes slowly inactivated, it is rather rapidly inactivated below pH 4.2. In the sedimentation experiments carried out below pH 4.2, $s_{20,w}$ is somewhat smaller than 12 S, but the experiments are difficult to interpret since the area under the peak decreases considerably with time. Enzyme inactivated at low pH values will precipitate when the pH of the solution is raised above 6.

Fig. 1 also shows values of $s_{20,w}$ obtained in 0.1 M phosphate buffer from pH 4.3 to 6. In phosphate, the enzyme also dissociates below pH 5, but the tendency to dissociate seems rather less than in Tris-acetate. The same observation is made at neutral pH values as a function of enzyme concentration, and it appears that phosphate ions have more tendency than acetate to prevent dissociation of the enzyme.

A number of different buffers have been tested for their effect on the sedimentation coefficient at alkaline pH values. Those that had little or no effect at pH 9.8 include glycine, phosphate, arsenate, borate, and Tris. At higher pH values, some dissociation was evident since the sedimentation coefficient did decrease slightly. On the other hand, the presence of a 0.2 M carbonate-bicarbonate buffer at pH 9.8 caused dissociation of the enzyme molecule into units of 250,000 molecular weight ($s_{20,w} = 12 S$). Results of sedimentation experiments from pH 7.5 to 10.2 in 0.2 M bicarbonate buffer solutions are shown in Fig. 2. The decrease in $s_{20,w}$ values as a function of pH may be described by a curve identical with a simple titration curve. In Fig. 2, the solid line is drawn with a pK value of 8.6. This dissociation is reversible provided that the enzyme has not become inactivated. As at pH 4.0 in Tris-acetate buffers, enzyme inactivated at alkaline pH values tends to aggregate and form a precipitate on lowering the pH toward neutrality.

Fig. 3 shows that the extent of dissociation of the enzyme at alkaline pH values depends upon the carbonate-bicarbonate concentration. In this figure, values of $s_{20,w}$ at pH 9.6 are plotted as a function of the carbonate-bicarbonate concentration. Clearly, at a buffer concentration of 0.02 M, the pK for dissociation would be considerably higher than 8.6. The presence of other buffers prevents dissociation. Thus, at pH 9.4, in 0.1 M glycine, $s_{20,w}$ is essentially unchanged in the presence of 0.2 M carbonate-bicarbonate buffer. It is apparent that the dissociation of the enzyme is rather specific for the particular carbonate-bicarbonate buffer system. The presence of 1,10-phenanthroline did not
cause any further dissociation of the enzyme at pH 9.6 in 0.2 M carbonate-bicarbonate buffer.

In an attempt to determine the effect of coenzyme on the dissociated enzyme in 0.2 M bicarbonate at pH 9.8, several experiments were performed in the presence of TPNH or DPNH. It was observed that the sedimentation coefficient had decreased from 12 S to a value of approximately 6 to 7 S in the presence of the reduced coenzymes. Further investigation showed the enzyme to become completely inactivated in a matter of minutes, even at 0°, in the presence of DPNH or TPNH. This result was rather surprising in view of the fact that it had been previously observed that high concentrations of coenzyme protected the enzyme against inactivation at pH 8 in Tris-acetate buffers (7). It appears, however, that this protection is pH-dependent and is dissociated into fragments of molecular weight lower than 250,000 is irreversibly inactivated. Reagents such as cysteine or EDTA do not protect against inactivation nor restore activity of the fragmented enzyme.

Effect of Temperature—Determinations of the change in the weight average molecular weight as a function of temperature at an enzyme concentration of 0.08 mg per ml. The original concentration of sodium dodecyl sulfate was 0.2 and 0.05% in the upper and lower cells, respectively. However, when KCl was added and the solutions cooled to 0°, some of the sodium dodecyl sulfate precipitated and was removed by centrifugation. The two peaks in each of the cells represent active and irreversibly inactivated enzyme. In the upper cell the two peaks have s20, w values of 20.9 and 6.05 S. Approximately 20% of the original activity of the enzyme remained. In the lower cell the two peaks have s20, w values of 25.2 and 8 S. Here about 60% of the activity remained. Thus, it is apparent again that enzyme that is dissociated into fragments of molecular weight lower than 250,000 is irreversibly inactivated. Reagents such as cysteine or EDTA do not protect against inactivation nor restore activity of the fragmented enzyme.

Table I shows typical values for the weight average molecular weight, as a function of temperature at an enzyme concentration of 0.08 mg per ml. It is clear that the slow moving peak (s20 < 5 S). Efforts to determine the effect of 3 M urea at low or high pH values invariably failed because of the rapid inactivation and precipitation of the enzyme. Inhibition by urea in 0.1 M phosphate buffer, pH 7.4, has been shown by Inagaki (8). In this case also, the enzyme is essentially completely inhibited by 3 M urea. Inagaki's results indicate that the urea is competitive with glutamate or with ammonium ion, but not with α-ketoglutarate.

Effect of Sodium Dodecyl Sulfate—Sodium dodecyl sulfate has been shown to cause dissociation of some proteins into subunits. The concentration required is fairly high and presumably micelles of the detergent are formed. Sodium dodecyl sulfate will also dissociate glutamic dehydrogenase into subunits of very low molecular weight. Fig. 4 shows the sedimentation pattern obtained with differing concentrations of sodium dodecyl sulfate at an enzyme concentration of 3.2 mg per ml. The original concentration of sodium dodecyl sulfate was 0.2 and 0.05% in the upper and lower cells, respectively. When KCl was added and the solutions cooled to 0°, some of the sodium dodecyl sulfate precipitated and was removed by centrifugation. The two peaks in each of the cells represent active and irreversibly inactivated enzyme. In the upper cell the two peaks have s20, w values of 20.9 and 6.05 S. Approximately 20% of the original activity of the enzyme remained. In the lower cell the two peaks have s20, w values of 25.2 and 8 S. Here about 60% of the activity remained. Thus, it is apparent again that enzyme that is dissociated into fragments of molecular weight lower than 250,000 is irreversibly inactivated. Reagents such as cysteine or EDTA do not protect against inactivation nor restore activity of the fragmented enzyme.

### Table I

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Apparent molecular weight × 10^4</th>
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<tbody>
<tr>
<td>11.5</td>
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<tr>
<td>15</td>
<td>5.3</td>
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<tr>
<td>18</td>
<td>5.18</td>
</tr>
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<td>24</td>
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</tr>
<tr>
<td>33</td>
<td>4.7</td>
</tr>
</tbody>
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higher temperatures favor dissociation of the enzyme. A mathematical analysis of dependence of the weight average molecular weight upon total enzyme concentration is exceedingly complicated for a monomer-dimer-trimer-tetramer system, and changes in the equilibrium constant with temperature are not easily obtained.

Above 40°, the turbidity begins to increase rapidly and visual observation shows that the solution becomes slightly turbid as a result of denaturation. This change is not reversed upon lowering the temperature.

**Discussion**

The results presented in this paper clearly show that there are at least two distinct types of dissociation of the glutamic dehydrogenase molecule. Defined in terms of the activity of the enzyme, these two types of dissociation may be classified, for the present, as reversible and irreversible dissociation. Reversible dissociation involves dissociation of enzyme of molecular weight 1,000,000 into 4 units of molecular weight 250,000. This type of dissociation occurs most simply by dilution, but in concentrated enzyme solutions is caused by urea (up to 3 M), 1,10-phenanthroline (2); thymoxine (9); phenanthridine (4); low pH; bicarbonate (above pH 8); various sterols in the presence of phenanthroline (2); thyroxine (9); phenanthridine (4); low pH; and even further (irreversible) dissociation of the molecule. These processes seemed to occur simultaneously. Similarly, the extent of dissociation into fragments by sodium dodecyl sulfate

Concerning the reversible dissociation, one is tempted to make at least two classifications of the methods for causing such dissociation, (a) those conditions that cause dissociation in the absence of coenzyme; and (b) those conditions that require the presence of coenzyme. Unfortunately, not much can be said about the mode of dissociation, i.e., whether the two classes represent a different kind of dissociation or whether any method of dissociation causes the same type of structural changes in the protein molecule. At first guess, it would seem unlikely that all the methods accomplish the dissociation in exactly the same way. Thus, dissociation at low pH may be the result of electrostatic repulsion, which is due to changes in the degree of ionization of groups in the enzyme molecule. Zine is known to be a constituent of the enzyme (10), and it has been claimed that zinc ions are removed from the enzyme by dialysis at low pH (11). Perhaps the dissociation at low pH is related to the removal of zinc from the enzyme molecule, but it is also possible that the enzyme must be inactivated before the zinc can be removed by dialysis. Dissociation by 1,10-phenanthroline was originally interpreted as indicating the importance of zinc in the association-dissociation reaction (2); but it is likely that dissociation with such compounds as phenanthridine or even 1,10-phenanthroline is unrelated to electrostatic effects or to zinc binding, but rather to disrupting hydrophobic bonds involved in linking subunits. That these effects are similar to those at low pH is indicated by the fact that 1,10-phenanthroline does not cause further dissociation at pH 4.3.

The cause of dissociation by bicarbonate-carbonate buffer at high pH is not clear. Since the presence of 1,10-phenanthroline at pH 9.5, in addition to 0.2 M bicarbonate, did not cause additional dissociation, the effects again are not independent of each other. In addition, one must assume that dissociation is related to the concentration of bicarbonate, rather than carbonate ion, since the pK for dissociation can occur appreciably below the pK of the buffer. Although it appears that the subunits formed upon reversible dissociation by the different methods outlined above are not substantially different, the results indicate the complexity of the forces involved in the association of the 4 units of 250,000 molecular weight.

It is to be noted that in all studies discussed here in which the reversible dissociation is forced in the absence of coenzyme, only one peak is observed in the sedimentation pattern. According to Gilbert (12), for a system in which polymers greater than dimers are formed, there should be more than one peak observed even if the equilibria are adjusted rapidly. However, it is possible that consecutive dimerizations, i.e. monomer to dimer, dimer to tetramer, would still yield only a single peak if the intermediate product were present in a significant amount. In experiments in which the dissociation is forced, the reactions to be considered may be monomer to dimer in some cases and dimer to tetramer in others. Thus the sedimentation pattern would appear only as a single peak even though the sedimentation coefficient is decreasing. In experiments in which the coenzymes are used to cause reversible dissociation, two peaks are usually observed (5). One could postulate that in these experiments the dimer concentration has been considerably decreased and that in the resulting monomer-tetramer equilibrium, two peaks are formed in accordance with the Gilbert theory.

It is of some interest that the presence of reduced coenzyme at pH 9.5 in 0.2 M bicarbonate buffer causes rapid inactivation and even further (irreversible) dissociation of the molecule. These processes seemed to occur simultaneously. Similarly, the extent of dissociation into fragments by sodium dodecyl sulfate
seems strictly related to the extent of irreversible inactivation of the enzyme. The results support the conclusion that the subunits of 250,000 molecular weight consist of a number of peptide chains which may be dissociated by methods that do not cause rupture of covalent bonds.

SUMMARY

The properties of crystalline beef liver glutamic dehydrogenase with respect to dissociation of the enzyme molecule have been investigated. It is shown that there are two distinct types of dissociation which result in subunits of markedly different molecular weight. One of these processes is a reversible dissociation in which 4 subunits of molecular weight 250,000 undergo a reversible association reaction to enzyme of molecular weight of 1,000,000. This type of dissociation may be affected by a large number of factors; in this paper it is shown to be enhanced by urea (up to 3 M), by lowering the pH to values between 4 and 5, by the presence of bicarbonate above pH 8, and by increasing temperatures between 10 and 35°. When dissociation of this type occurs, enzymatic activity is decreased but may be restored by removing the conditions leading to the dissociation.

The second type of dissociation is irreversible both with respect to regaining molecular weight species in the region of 250,000 to 1,000,000 and restoring enzymatic activity when conditions causing this type of dissociation are removed. This irreversible dissociation may be induced by the presence of sodium dodecyl sulfate, by 6 M urea, or by the presence of reduced coenzyme in bicarbonate buffers at pH values above 8.5. Examination of this dissociated material in the ultracentrifuge indicates that it is of low molecular weight (30,000 to 60,000). It is concluded that the subunit of 250,000 molecular weight consists of a number of peptide chains (four to eight) which are held together by bonds other than covalent bonds. In every case studied, disrupting these bonds was accompanied by irreversible inactivation of the enzyme.

Addendum While this paper was in preparation, a paper by Wolff (13) appeared in which the effect of thyroxine on the sedimentation coefficient of glutamic dehydrogenase was examined in some detail. It was also shown that thyroxine and several thyroxine derivatives as well as CNS−, I−, or ClO4− were effective in causing reversible dissociation. On the other hand, Wolff also observed that 5 M urea and sodium dodecyl sulfate resulted in the appearance of a slow moving peak in the ultracentrifuge. Also, Jirgensons (14) has shown that treatment of the enzyme with sodium dodecyl sulfate gives rise to material with a low sedimentation coefficient and that the enzyme contains a large number (18 to 25) of NH2-terminal amino acid residues per 106 molecular weight. The molecular weight of sodium dodecyl sulfate-treated enzyme was calculated as 43,000.

REFERENCES

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