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Acetoacetate Decarboxylase. Reassociation of Subunits*

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ABSTRACT: The enzyme acetoacetate decarboxylase can be dissociated into monomeric subunits in acid, urea, or guanidium chloride solution, but these subunits cannot be reassociated by any method so far known. However, at pH 8 in 4 M urea solution at low temperature, the enzyme is dissociated into subunit dimers, and these subunit dimers can be reassociated to form native, active enzyme simply by diluting the urea solution with phosphate buffer at pH 5.96 in the presence of dithiothreitol.

The enzyme acetoacetate decarboxylase consists of twelve subunits of molecular weight about 29,000 each (Tagaki and Westheimer, 1968). Prior research (Lederer *et al.*, 1966) had shown that the enzyme can be dissociated into subunits by acid, urea, guanidium chloride solution, or sodium dodecyl sulfate, but these subunits are enzymically inactive, and no method has yet been found to cause these subunits to reassociate to active enzyme. However, at pH 8 and in 2.5–4 M urea solution at 0° the enzyme is dissociated into particles composed of two subunits, and these subunit dimers can be reassociated into active enzyme. The reassociated enzyme has been crystallized, and appears identical with native in crystal form and ultraviolet spectrum. The details of this reassociation are given here.

Experimental Section

Materials. Crystalline acetoacetate decarboxylase was prepared by the method of Zerner *et al.* (1966); it was recrystallized prior to use and made up into a stock solution in 0.05 M phosphate buffer (pH 5.92). For determinations of sedimentation velocity at pH 8, the stock solution was dialyzed against 0.05 M sodium phosphate buffer containing 0.01 M dithiothreitol. Urea (Merck) was used without recrystallization, but was dissolved in buffer just prior to use. A 4 M solution of urea was analyzed for cyanate, and within the limits of Werner's method (Werner, 1923; *cf.* Stark *et al.*, 1960) showed none; the concentration of cyanate was therefore less than 2×10^{-3} M. In control experiments, the enzyme activity was found to be unaffected by this concentration of cyanate. *N*-Ethylmorpholine (Union Carbide) was distilled before use; dithiothreitol (Calbiochem) and β-mercaptoethanol (Eastman Kodak) were used without further purification. Acetopyruvic acid was prepared as described elsewhere (Tagaki *et al.*, 1968).

Assay. The method of assay is critical for this work. The usual method (Fridovich, 1963; Warren *et al.*, 1966) involves pipetting 0.1 ml of enzyme solution and 0.3 ml of a 0.3 M solution of lithium acetoacetate into 2.6 ml of 0.1 M phosphate buffer (pH 5.92) at 30°, and observing the decrease in optical density at 270 mμ. This method dilutes the enzyme solution 30-fold and, when it is in urea solution, also dilutes the urea 30-fold. Measurements are taken every 15 sec in a Zeiss PMQ-II spectrophotometer or continuously in a Cary 15 spectrophotometer, and the change in optical density, in ordinary cases, is linear with time over the first 120 sec. In the present investigation, reassociation sometimes occurred during the assay, so that the plot of optical density against time was not linear. The assay was continued until a linear portion of the curve was obtained; this usually required about 4–6 min.

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TABLE I: Sedimentation Velocity for Enzyme, Reassociated Enzyme, and Subunits.

pH	Enzyme Concn (mg/ml)	Urea Concn (M)	Prior Treatment	Enzyme Act. (%)	Sedimentation Constant (S)
5.98	2.0	0.00	Native enzyme	100	13.1[b]
5.98	4.92	4.0	25 hr at 4°	12	0.78[c] (major) 6.30[c] (minor)
8.00	4.92	4.0[a]	25 hr at 4°	63	1.78[c]
5.98	2.0	0.00	Recovd by dialysis from soln above and diluted with buffer	74	13.2[b]

[a] With 1.0×10^{-2} dithiothreitol added. [b] 20°. [c] 9°.

Ultracentrifugation. Sedimentation velocity experiments were carried out with a Beckman–Spinco Model E analytical ultracentrifuge, using 12-mm, 4° sector centerpieces and schlieren optics; both normal and wedge cells were used (Schachman, 1959; Lederer *et al.*, 1966). The composition of solutions used and the observations are given in Table I.

In some experiments in 4 M urea, part of the reaction mixture was set aside prior to ultracentrifugation, and maintained at 0°. The reserve solution was then combined with that recovered from the corresponding ultracentrifugation cells, and dialyzed at 4° against 0.05 M phosphate buffer (pH 5.98) for 24 hr with two changes of the external solution. The protein exposed to urea at pH 5.98 largely precipitated and the residual soluble protein had a low specific activity. The protein exposed to urea at pH 8.10 remained in solution. It was returned to the ultracentrifuge cell, after some dilution, and the data in Table I were obtained.

Reassociation of Enzyme. Enzyme solutions (1–5 mg/ml in 0.1 M (pH 8.0) *N*-ethylmorpholine–*N*-ethylmorpholinium sulfate buffers or sodium phosphate buffer) and 4.0 M urea were maintained at 4° for 24 hr, and then dialyzed against 0.05 M (pH 5.92) phosphate buffer at 4° for 24 hr. The resulting enzyme solution showed high specific activity, usually 70–85% of that of the control. The ultraviolet spectrum of the enzyme so produced was indistinguishable from that of native enzyme. In particular, in the urea solutions the unexplained absorption band at 320 mμ (Lederer *et al.*, 1966; Tagaki *et al.*, 1968) was diminished and less sharp, but it was completely restored in the reassembled enzyme. Furthermore, the recovered enzyme could be crystallized by the usual procedure (Hamilton and Westheimer, 1959; Zerner *et al.*, 1966), and the resulting crystals proved indistinguishable in shape and character from those of native enzyme.

Dissociation in 4 M Urea, pH 8, and 5°. A measured volume (0.05 ml) of an enzyme stock solution (9.8 mg/ml, specific activity 45.3) was added at 5.0 ±

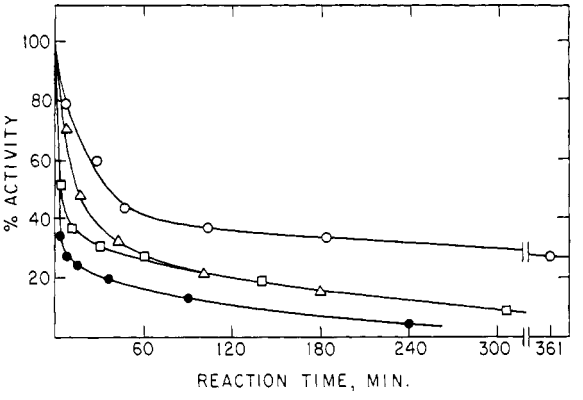

FIGURE 1: Effect of pH and urea on the inactivation of acetoacetate decarboxylase at 25°. After the indicated time in 4 M urea, the enzyme solutions were diluted with 30 volumes of pH 6 buffer and incubated for 15 min prior to assay (*i.e.*, these are maximum rates, after partial recovery of activity). (○) pH 5.90, (△) pH 4.62, (□) pH 6.94, and (●) pH 8.04.

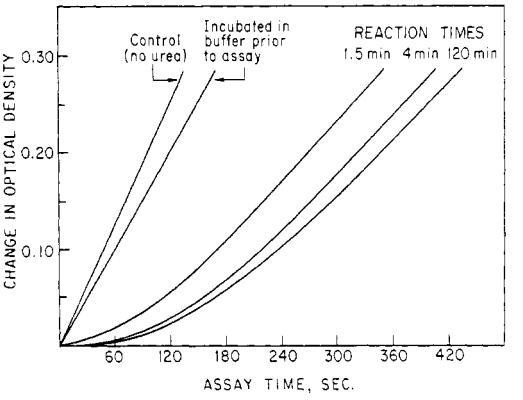

FIGURE 2: Assay of acetoacetate decarboxylase that has been treated at 4° with 4 M urea in 0.1 M *N*-ethylmorpholine–*N*-ethylmorpholinium sulfate buffer (pH 8.10) in the presence of 1.0×10^{-3} M dithiothreitol. The lower straight line refers to the assay of a sample that had stood in solution in 4 M urea at 4° for 77 min, and then had been incubated at 30° in 0.05 M phosphate buffer (pH 5.98) prior to assay. Note that the assay curves of the samples that had not been incubated in buffer start with zero slope.

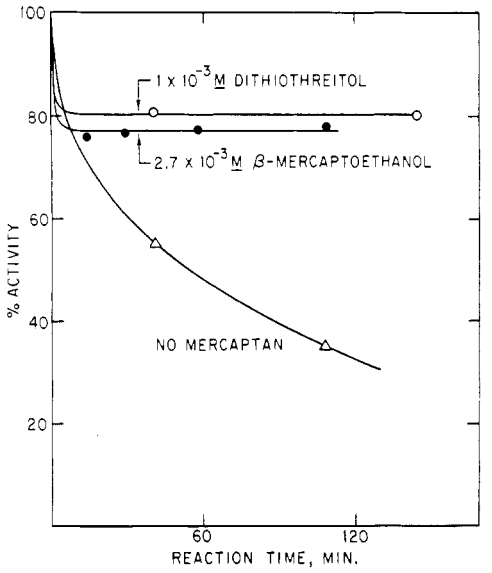

FIGURE 3: Per cent of activity recovered from enzyme treated for the "reaction time" shown with 4 M urea in a 0.1 M *N*-ethylmorpholine–*N*-ethylmorpholinium sulfate buffer (pH 8.10) at 4°, with and without mercaptans added. The enzyme solution was diluted 30-fold with pH 6 buffer and incubated for 15 min in this buffer prior to assay.

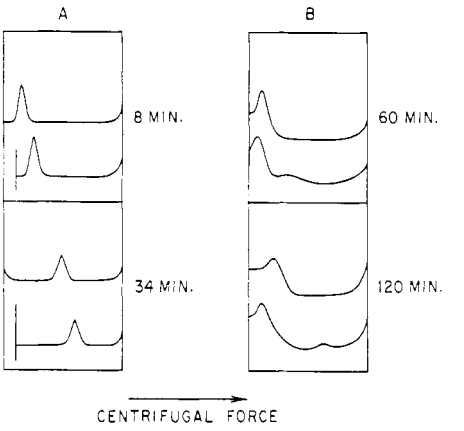

FIGURE 4: Sedimentation velocity experiments (at 50,470 rpm) showing native, dissociated, and reassembled enzyme. (A) Upper line, native enzyme, s_{obsd} = 13.1 S. Lower line, reassembled enzyme, s_{obsd} = 13.1 S. Temperature of measurement, 20°. (B) Upper line, enzyme at 4 M urea at pH 8 with 0.01 M dithiothreitol, s_{obsd} = 1.78 S. Lower line, enzyme in 4 M urea at pH 6, s_{obsd} = 0.78 S and (in trace amounts) s_{obsd} = 6.3 S. Temperature of measurements, 9°.

0.3° to 1.95 ml of 4.1 M urea in 0.1 M *N*-ethylmorpholine–*N*-ethylmorpholinium sulfate buffer (pH 7.86). The pH of the resulting solution, measured at 25°, was 8.10. Aliquots (0.05 ml) were withdrawn with a Hamilton syringe and assayed in 2.9 ml of the usual assay solution at 30°, using a Cary spectrophotometer with slide wire for full deflection at 0.1 ODU. In a second type of experiment, similar aliquots were incubated for 15–20 min at 30° in 2.6 ml of 0.05 M potassium phosphate buffer (pH 5.98); subsequent to the incubation, the solutions were assayed by adding 0.3 ml of 0.3 M lithium acetoacetate solution to the incubated mixture. In other assays, the methods were the same except that the urea solutions contained 10^{-2}–10^{-3} M β-mercaptoethanol or dithiothreitol.

Results

Evidence for Reactivation. The enzyme is irreversibly denatured by 4 M urea at 25°. The effect of pH and time of incubation in the urea solution on this denaturation is shown in Figure 1. The denaturation is more rapid and more extensive at pH 8 than at pH 6. When the assays are conducted with solutions exposed to 4 M urea at pH 6, they yield normal curves, *i.e.*, the change in optical density is linear with time, at least for the first several minutes of the assay (eventually the rate falls when the acetoacetate is largely consumed). However, when the assays are conducted at pH 6 with solutions of enzyme previously exposed at pH 8 to 4 M urea at 4°, the rate is initially zero; that is to say, the plot of optical density against time is curved where the slope of the curve is initially zero and subsequently rises during the assay. This is an essential point in the argument; it demonstrates that the species present in 4 M urea at pH 8 is inactive, but when the urea concentration is lowered for assay, a change occurs that leads to reactivation (Figure 2). If the enzyme solution after treatment with urea is diluted 30-fold with pH 5.92 buffer, and then is incubated for 15 min in this buffer prior to initiating the assay, then the plot of optical density against time is linear. The recovery of activity reaches an upper limit after 15-min incubation in buffer; longer times do not appreciably increase the enzymic activity.

Conditions for Reactivation. All attempts at reactivation of enzyme exposed to urea at pH 6 failed, and reactivation has succeeded only with enzyme exposed to urea at pH 8 and at low temperatures; as shown in Figure 1, at 25° the enzyme is irreversibly inactivated regardless of pH. At low temperatures, however, the final activity recovered is essentially independent of the time that the solution is allowed to remain at 5° in 4 M urea at pH 8 prior to dilution and incubation at pH 6. Figure 3 shows that, in the presence of dithiothreitol or β-mercaptoethanol, about 80% of enzymic activity can be recovered for enzyme that has stood for almost 2 hr in 4 M urea, provided that the enzyme solution is incubated at 30° in pH 6 buffer for 15 min prior to assay. On the other hand, in the absence of any mercaptan, the loss of enzymic activity on standing in 4 M urea, even at 5°, is progressive with time and irreversible as also shown in Figure 3. In the presence of dithiothreitol, good recoveries of enzymic activity (75–85%) can be obtained even from solutions of enzyme that have remained for 24 hr in 4 M urea solution at 4°.

Sedimentation Velocity. The data obtained by sedimentation velocity experiments are presented in Table I. Figure 4 presents tracings of the schlieren lines in

ultracentrifugation. The frames marked A show the sedimentation for native enzyme and reconstituted enzyme; the values of s_{obsd} are essentially identical. The frames marked B were determined in 4 M urea at pH 6 and 8. At the lower pH, the only species present has an s_{obsd} of 0.78 S, and is presumably the ultimate, monomeric subunit of acetoacetate decarboxylase. A small amount of another species, with s_{obsd} = 6.3 S, is also present, but has not been conclusively identified; the possibility that it is native enzyme has not been ruled out, since its s value could be strongly affected by urea, both because of the change is density of the solutions and the change in shape of molecule. At pH 8, however, the only species present has an s_{obsd} of 1.78 S. This must be some species other than monomer; the ultracentrifugal measurements reported in an accompanying article (Tagaki and Westheimer, 1968) show that this material consists of dimers of subunits. These crude values of the sedimentation constants have not been corrected for solvent density or viscosity but are at least qualitatively significant.

Discussion

Prior research (Lederer *et al.*, 1966) had shown that the enzyme acetoacetate decarboxylase dissociates into subunits at pH 2 in 8 M urea and in guanidinium chloride solution. Despite repeated attempts, reassociation of the enzyme from these solutions has been unsuccessful. It has here been shown, however, that the enzyme can be dissociated, presumably into dimers of the subunit, at pH 8 in 4 M urea at 5°. The enzyme is initially without enzymic activity under the assay conditions; this conclusion rests on the observation (see Figure 2) that the initial slope of the assay curve is zero. The enzyme can be reassociated and slowly reactivated by removing the urea by dialysis against a buffer at pH 6, or more simply by diluting the urea 30-fold in buffer at pH 6, provided that an effective mercaptan (β-mercaptoethanol or dithiothreitol) is present.

The evidence for these statements is as follows. The inactivation and reactivation of the enzyme is amply demonstrated in the experiments shown in Figures 2 and 3, and the need for dithiothreitol or β-mercaptoethanol is illustrated in Figure 3. In particular, it is important to note that, in 4 M urea at 5°, the enzyme is rapidly inactivated, so that after only 1.5 min under these conditions, the activity has been reduced to zero; this fact is illustrated by the initial value of zero of the slope of the plot of optical density against time in the assays illustrated in Figure 2.

The question must naturally arise as to whether the enzyme is dissociated under these conditions, or whether it has been inactivated by some other process. The dissociation to dimers has been established in two ways. First, the sedimentation velocity experiments, recorded in this paper, show that the native enzyme (s_{obsd} = 13 S) is entirely absent from 4 M urea solution at pH 8 and 4°; the only species present is one with a much lower sedimentation constant (s_{obsd} = 1.78 S). Further, this material is not the same as the monomeric subunits, which have an s_{obsd} = 0.78 S. The intermediate value, 1.78 S, of the sedimentation constant is most simply interpreted as dimer. More quantitative data are presented in the accompanying paper (Tagaki and Westheimer, 1968). The molecular weight of the monomeric subunit, determined by the Yphantis method (Yphantis, 1964), is there shown to be about 29,400. In 2.5 M urea at 10° the molecular weight of the protein is about 60,000. Thus in urea solutions similar (although not identical with) those here used, the molecular weight of the dominant species present is that of a dimer. The identical experimental conditions were not used for the ultracentrifugal experiments by the Yphantis method as for those here reported for a minor technical reason; it was not practical to operate the ultracentrifuge at a low enough temperature to prevent irreversible denaturation of the enzyme in 4 M urea over the long periods of time required for the determination of molecular weights. (The sensitivity of the enzyme solutions at pH 8 in 4 M urea to higher temperatures has already been discussed.) Nevertheless, the data were obtained under conditions sufficiently close to those used here as to leave little doubt that the species of s_{obsd} 1.78 S found in sedimentation velocity experiments is in fact a dimer of subunits. It is this dimer that has been reassembled to active enzyme, indistinguishable from native acetoacetate decarboxylase.

Acknowledgments

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