Uricase. Subunit Composition and Resistance to Denaturants†

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ABSTRACT: The native molecular weight of pig liver uricase as estimated by sedimentation equilibrium is 125,000. Gel filtration chromatography and sedimentation velocity indicated a globular shape for the enzyme. Uricase exhibited unusual resistance to denaturation by either sodium dodecyl sulfate or guanidinium chloride. Extensive treatment with either denaturant allowed molecular weight estimates by three different methods, all of which indicated the enzyme is composed of four subunits of 32,000 molecular weight. Inhibition kinetics suggest that the resistance of uricase to guanidinium chloride denaturation is caused, in part, by the denaturant assuming a substrate analog protective role. Thermal inactivation studies revealed at least two and possibly three kinetically distinguishable processes.

Uricase, urate:oxygen oxidoreductase (EC 1.7.3.3), has been localized intracellularly within a crystalloid structure of the microbodies (Hruban and Rechcigl, 1969). To understand better the nature of intracellular uricase and the regulation of its activity, it was deemed first necessary to investigate the properties of this enzyme in solution. Mahler *et al.* (1955) estimated the native molecular weight of uricase by sedimentation velocity at a single protein concentration and by metal content. Since these estimates possessed relatively wide error ranges, a reinvestigation of the native molecular weight of uricase and a determination of its subunit composition were undertaken. During the course of this investigation, uricase was observed to exhibit a somewhat unusual resistance to the commonly employed denaturants; these observations are also reported.

Experimental Procedures

Pig liver uricase was obtained by special order from Boehringer-Mannheim Corp. as a solution containing 2 mg/ml of protein in 50% glycerol, buffered to pH 10.2 with glycine and sodium carbonate. The specific activity of these preparations was 16.4 IU/mg when assayed spectrophotometrically for intermediate appearance at 312 nm (Priest and Pitts, 1972; Pitts and Priest, 1973). A molar absorption difference of 4850 was used. Solution conditions for these assays were 1 mM urate buffered to pH 8.8 with 0.10 M glycine at 25° in an open cuvet. Protein concentrations were estimated from absorbance at 276 nm using an $\epsilon_{276}^{1\%}$ of 11.3 as reported by Mahler (1963).

Polyacrylamide disc gel electrophoresis of the native enzyme was conducted according to the method of Ornstein (1964) and Davis (1964), and in the presence of sodium dodecyl sulfate by the method of Weber and Osborn (1969). Protein was typically stained with Coomassie Blue, and gels were scanned at 550 nm on a Beckman Acta CIII. Copper was determined by the neutron activation method (Guinn and Lukens, 1965).

Sedimentation velocity and equilibrium experiments were performed at 25° in a Beckman Model E analytical ultracentrifuge equipped with schlieren and interference optics. The solvents employed for velocity and equilibrium measurements on native uricase were 0.1 M glycine buffers, pH 10.2 and 9.0, respectively. The experimentally determined values for solvent density and relative viscosity at 25° were 1.0024 g/ml and 1.0203, respectively. Equilibrium measurements at 13,000–18,000 rpm employed the short-column meniscus depletion method (Yphantis, 1964) or the long-column meniscus depletion method described by Chervenka (1970). Initial protein concentrations were between 0.2 and 0.5 mg/ml. Equilibrium times were estimated from the semiempirical equation given by Yphantis (1964), and attainment of equilibrium was verified by measuring the fringe displacement at several radial distances on exposures taken 4 hr apart. Equilibrium measurements in 6 M guanidinium chloride (Heico, Inc., Delaware Water Gap, Pa.) were obtained with the long-column meniscus depletion method (Chervenka, 1970). Protein solutions in 6 M guanidinium chloride were dialyzed against this solvent at least 5 days at 25° prior to ultracentrifugation. Sedimentation velocity measurements at pH 10.2 were performed on uricase at concentrations of 1–4 mg/ml. The solubility characteristics of uricase at pH 9.0 prevent velocity measurements at concentrations higher than about 2 mg/ml. A partial specific volume of 0.73 ml/g was estimated for uricase from its amino acid composition. The effective partial specific volume in guanidinium chloride was assumed to be the same as the partial specific volume in dilute salt solution.

Gel chromatography of native uricase was performed on a 2.0 × 100 cm column of Sephadex G-200 in 0.1 M glycine (pH 10). The column was calibrated with proteins of known equivalent hydrodynamic radii, R_e, calculated from literature values of their diffusion coefficients (Tanford, 1951). R_e was expressed as a function of elution position according to the method described by Ackers (1967). Gel chromatography in 6 M guanidinium chloride of reduced and carboxymethylated uricase was performed as described by Mann and Fish (1972).

When using empirical methods such as sodium dodecyl sulfate gel electrophoresis or gel filtration in guanidinium chloride on proteins of unknown properties, we prefer to use the term "apparent R_e" to describe an electrophoretic band or gel elution zone until the actual mass of the species is verified by an independent measurement. If the standard polypeptide chains employed have been reduced so that none are constrained by disulfide bonds, the following relations may be used to calculate R_e: for linear polypeptide chains in 6 M guanidinium chloride[1]

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[1] This function is derived from the relationship given by Tanford (1968).

$$R_e = 2.306(n)^{0.555}\ \text{Å} \quad (1)$$

for linear polypeptide–sodium dodecyl sulfate complexes[2]

$$R_e = 0.682(n)^{0.751}\ \text{Å} \quad (2)$$

where n is the number of amino acids in the polypeptide chain. For sodium dodecyl sulfate gel electrophoresis, log R_e is plotted *vs.* mobility; gel chromatography data are treated as described previously (Ackers, 1967).

Results

The uricase preparation used in these studies eluted from a Sephadex G-200 gel chromatography column as a single symmetrical zone of 280-nm absorbing material and enzyme activity. The specific activity of the enzyme was constant across the elution zone. Polyacrylamide gel electrophoresis at pH 9.5 yielded a single, although somewhat broad, protein-stained band. As shown in Figure 1, the uricase activity profile from a sectioned gel is congruent with the zones of stained protein in companion gels.

The specific activity of the uricase preparation could not be increased by repeated precipitation from distilled water or by preincubation for 30 min in the presence of 1.0 mM concentrations of CuCl, $CuCl_2$, $FeSO_4$, $FeCl_3$, or $ZnCl_2$.

Mahler (1963) reported the presence of copper in uricase at a ratio of one metal atom per molecule of enzyme of 120,000 mol wt. The presence of copper was confirmed by neutron activation analysis and no manganese or zinc could be detected. When a sample of uricase was dialyzed for several days against glass distilled water to remove nonspecifically bound metal ions, the specific activity of the enzyme dropped from 16.4 to 8.1 units/mg. Quantitative copper analysis of an identical sample showed the presence of copper in a molar ratio of 0.52 mol of copper/mol of enzyme of mol wt 125,000. Thus, while copper was apparently lost during dialysis, a molar ratio of 1:1 for copper and uricase is estimated on the basis of the highest specific activity which could be obtained.

Due to the marginal solubility of uricase, sedimentation coefficients of the enzyme could be determined only in the concentration range of 1–4 mg/ml. This limited the precision of our data. The $s_{20,w}$ of uricase exhibited a positive dependence on concentration, characteristic of an associating–dissociating system. An $s^0_{20,w}$ of 6.8 S was estimated for uricase from these data. All experimentally measured sedimentation coefficients for uricase were significantly larger than the 5.5 S at 2 mg/ml reported by Mahler *et al.* (1955).

Short-column meniscus depletion sedimentation equilibrium runs on native uricase yielded linear plots of ln fringe displacement *vs.* r^2 for as much of the solution column as could be read. The weight average molecular weight for native uricase by this method was 125,500. Long-column meniscus depletion runs at slower speeds yielded slightly curved plots. For a typical run, the weight average over the whole cell was 131,500 and the Z-average molecular weight as estimated from the fringe displacement at the bottom of the solution column where the protein concentration was approximately 1 mg/ml was 155,000. Linear extrapolation of point slopes of ln fringe displacement *vs.* r^2 to zero fringe displacement (Yphantis, 1964) yielded an estimate of 124,800 for the mol wt of the species near the upper meniscus.

When the sedimentation coefficient of 6.8 S was combined with a mol wt of 125,000, an f/f_{min} of 1.34 was obtained for native uricase. This value is in acceptable agreement with an f/f_{min} of 1.29 obtained from the native molecular weight and an R_e of 42.7 Å by gel chromatography of native uricase. These data indicate that the gross conformation of uricase is that of a globular protein.

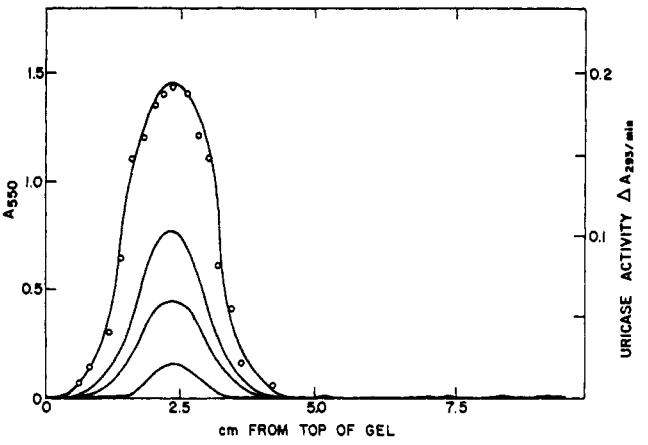

FIGURE 1: Disc gel electrophoresis of native porcine liver uricase. Solid lines are scans at 550 nm of 50-, 20-, 10-, and 2 μg samples of protein after electrophoresis and staining. Open circles represent uricase activity from 2-mm slices of unstained gel to which 50 μg of protein had been applied. Slices were incubated in buffer overnight and then assayed for uricase activity at 293 nm using 0.2 M borate buffer (pH 9.5) containing 1000 μM urate.

When uricase was initially placed in the presence of sodium dodecyl sulfate, three electrophoretic bands were observed. The most intensely stained band had an apparent R_e of 48 Å, while a prominently stained zone of apparent R_e = 82–92 Å and minor (<2%) bands of apparent $R_e > 96$ Å were also observed. As shown in Figure 2, upon heating in sodium dodecyl sulfate in the presence or absence of β-mercaptoethanol, the bands of apparent R_e = 82–92 Å and $R_e > 96$ Å disappeared with a concomitant increase in the band of R_e = 48 Å. An effective hydrodynamic radius of 48 Å would be exhibited by a 32,000 mol wt linear polypeptide in a sodium dodecyl sulfate–protein complex. The atypical times of heating required to effect almost complete conversion of uricase in sodium dodecyl sulfate to the R_e = 48 Å species suggests that the protein is unusually resistant to this denaturant.

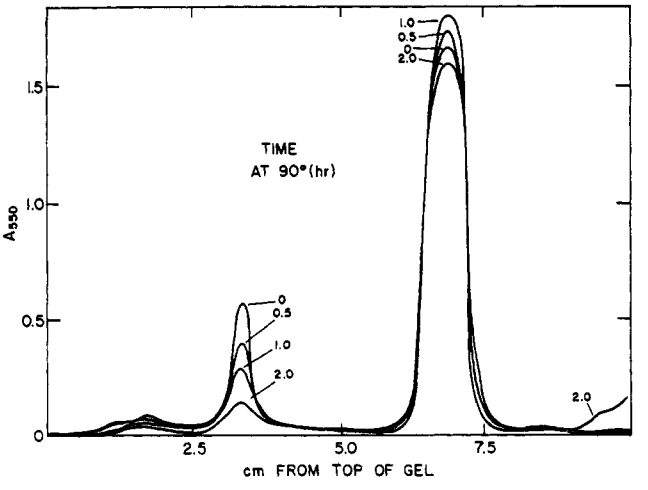

FIGURE 2: Sodium dodecyl sulfate gel electrophoresis of porcine liver uricase. Uricase samples contained 0.4 mg/ml of protein in 0.01 M phosphate (pH 8.0) with 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. Aliquots (20 μg) were taken for electrophoresis after incubation at 90° for 0, 0.5, 1.0, and 2.0 hr. Scans of the samples at 550 nm are superimposed.

[2] This function is derived from the relationship given by Reynolds and Tanford (1970).

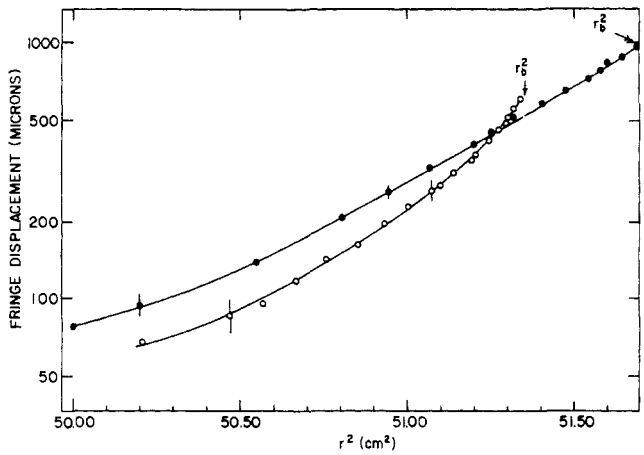

FIGURE 3: Sedimentation equilibrium of uricase in 6 M guanidinium chloride. (○) This sample contained 0.5 mg/ml of protein in 0.01 M acetate buffer (pH 4.75), 6 M guanidinium chloride, and 0.1 M iodoacetamide. The sample was dialyzed against the solvent at 25° for 5 days prior to centrifugation at 32,000 rpm. (●) Conditions for this sample were the same as above except the sample was heated for 1 hr at 78° before dialysis at 25° for 5 days. Vertical bars indicate maximum deviations incurred reading five fringes. Arrows indicate the bottoms of the solution columns.

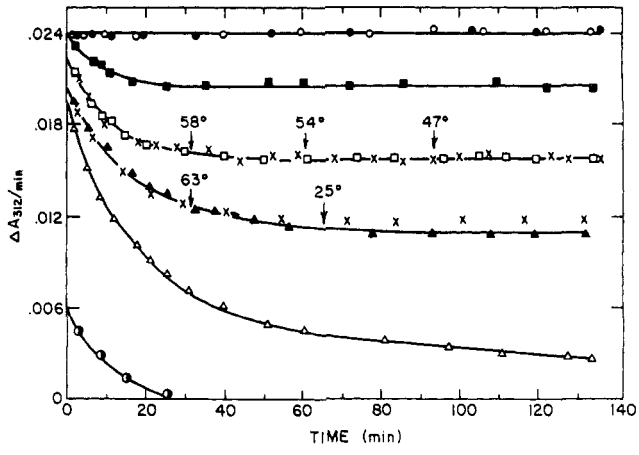

FIGURE 5: Thermal inactivation of uricase. Uricase solutions (0.5 mg/ml) were dialyzed for 24 hr against 0.1 M glycine (pH 8.8) buffer. Aliquots (1.0 ml) were immersed in a water bath maintained at 25° (○), 47° (●), 54° (■), 58° (□), 63° (▲), 68° (△), and 78° (◑). One minute after the desired temperature was attained 25 μl was removed and assayed at 312 nm, 25°, in 0.1 M glycine buffer (pH 8.8) containing 200 μM urate. The X indicates typical experiments in which renaturation was attempted by temperature lowering. Labeled arrows show time of attainment of a lower temperature.

The resistance of uricase to the disruptive effects of guanidinium chloride is illustrated in Figure 3. Sedimentation equilibrium of uricase after exposure to 6 M guanidinium chloride at 25° for at least 5 days produced a markedly curved plot. The limiting slope near the solution meniscus yielded a mol wt of ~32,000. The *Z*-average mol wt of the sample as estimated from the data at the bottom of the solution column was 120,000. Heating uricase in 6 M guanidinium chloride at 78° for 1 hr, at pH 4.7 prior to dialysis at 25° for 5 days, significantly diminished the curvature at either pH (Figure 3). Again the mol wt estimated near the upper meniscus was 32,000. The presence of reducing agent or a pH of 8.6 in the sample during heating and subsequent dialysis in 6 M guanidinium chloride did not abolish curvature in the sedimentation equilibrium data, and, in fact, yielded data quite similar to those obtained in the absence of reducing agent.

Gel chromatography of uricase which was reduced overnight in 6 M guanidinium chloride and carboxymethylated before application to the gel chromatogrphy column yielded small amounts of material of apparent R_e = 54 Å (mol wt 32,000).

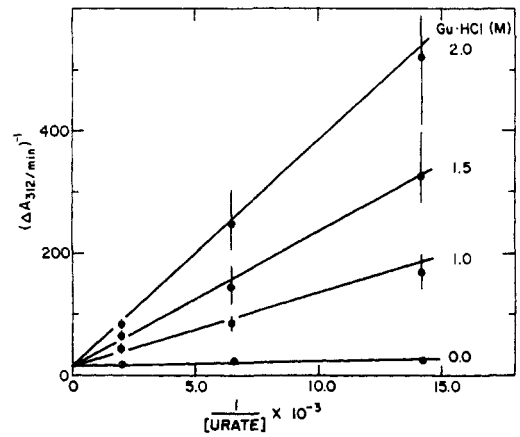

FIGURE 4: Competitive inhibition of uricase by guanidinium chloride. Assays were conducted at 25° in 3.0-ml reaction cells containing 0.1 M glycine buffer at pH 8.8. Reactions were initiated with 25 μg of uricase.

Although only a fraction of the material subjected to reduction and carboxymethylation was accounted for in the 54-Å peak, no other species could be detected. It is believed, though not quantitatively proven, that the remainder of the uricase was adsorbed on the walls of the collodion bag used to concentrate the sample just prior to its application to the column.

The data from three different methods in two different denaturing solvent systems strongly suggest that uricase is resistant to disruption by these commonly employed denaturants. That the resistance of uricase to guanidinium chloride denaturation might be due in part to a substrate analog "protective" role by the denaturant was considered in view of the structural similarity between the guanidinium ion and urate, a substrate for uricase.

Figure 4 shows that indeed guanidinium chloride behaves as a classical competitive inhibitor of the enzymatic oxidation of urate by uricase. Rajagopalan *et al.* (1961) have demonstrated that urea is also a competitive inhibitor of uricase. While guanidinium chloride has a relatively low inhibitory efficiency, nevertheless, at a concentration of 6 M, as was used in the subunit dissociation experiments, the enzyme would be expected to be saturated.

In addition to the chemical denaturants, uricase was subjected to temperatures up to 78°. Small (1.0 ml) samples were rapidly raised to the desired temperature and maintained there. Aliquots (25 μl) were withdrawn and assayed at 312 nm in 3.0-ml reaction cells at 25°. As can be seen in Figure 5, no reduction in uricase activity was observed up to 47°. At higher temperatures, activity decreased significantly, and this decrease was a function of both time and temperature. There are at least two and possibly three kinetic processes that can be observed. It can be seen in Figure 6 that data observed over the first 30 min shown in Figure 5 are linearized when a reversible two-state kinetic model is assumed. The apparent first-order rate constants obtained are shown in Table I. It is also apparent in Figure 5, however, that the process is in fact not reversible under the conditions of our experiments. At the higher temperatures it can readily be seen that activities continue to decrease and do not truly attain a constant level. This continued activity decrease was confirmed when samples

TABLE I: Temperature Dependence of Apparent First Order Rate Constants for Uricase Inactivation.

T (°C)	k_f (min^{-1})	k_b (min^{-1})
54	0.025	0.171
58	0.029	0.060
63	0.032	0.029
68	0.034	0.006

were held at each temperature overnight. It can also be seen in Figures 5 and 6 that the time course data do not extrapolate to the same initial point even though all data points were corrected for the approximately 1.5 min required to attain the desired temperature. This may indicate a relatively rapid temperature-dependent kinetic process; however, further study utilizing more rapid observation techniques will be required for verification and quantitation.

Discussion

Intracellularly, uricase is reported to be localized in a crystalloid structure within microbodies (Hruban and Rechcigl, 1969). On this basis, it might be expected that uricase would exhibit solubility properties different from those of typical globular proteins. This has been shown to be the case, since uricase is only sparingly soluble near neutral pH in the usual buffer systems (London and Hudson, 1956). Its solubility increases significantly only when the pH of the medium is increased to pH 9–10. Physical measurements conducted between pH 9 and 10 were done without concern about the effect of such basic conditions on protein structure, since uricase exhibits maximal activity near pH 9 (Baum *et al.*, 1956).

Repeated attempts to enhance the specific activity of the enzyme preparation through gel chromatography, reprecipitation, and gel electrophoresis produced no additional purification. Based on these criteria, it was presumed that the purity of our uricase preparation was sufficient to allow accurate interpretations of our molecular weight and polypeptide chain composition studies.

Although some aggregation of native uricase at pH 9 is observed by sedimentation equilibrium, extrapolation of data near the upper meniscus at low speeds or runs at higher speeds gives a mol wt estimate for the monomeric native enzyme of 125,000. This agrees reasonably well with the estimate of 120,000, by Mahler *et al.* (1955), based on metal content. We cannot explain the large discrepancy between our extrapolated sedimentation coefficient of 6.8 S and Mahler's reported value of 5.5 S (Mahler, 1963) at a finite protein concentration. The facts that our gel chromatography results (which were obtained at a uricase concentration quite similar to Mahler's) agree closely with our $s^0_{20,w}$ determined for uricase and that we observed a positive dependence of the sedimentation coefficient upon concentration argue against a greater degree of protein association under the conditions of our velocity experiments. A protein of 5.5 S would have to be of mol wt 90,600 and have $f/f_{min} = 1.44$ to exhibit an R_e of 42.7 Å by gel chromatography. The Mahler (1963) measurements were made on a uricase preparation of somewhat lesser reported specific activity than our preparation; however, since different assay methods were employed, a direct comparison is difficult.

The complete dissociation of uricase into its constituent polypeptide chains by the two common denaturants, sodium dodecyl sulfate and guanidinium chloride, presented some difficulty. It was necessary to heat the enzyme in the presence of sodium dodecyl sulfate for uncommonly long periods of time to effect complete dissociation. Some apparent fragmentation began to occur during the longest period of heating (Figure 3). We believe the transient sodium dodecyl sulfate electrophoretic component of apparent R_e = 82–92 Å to be undissociated uricase. Although the R_e of native uricase, 43 Å, is similar to that of a 32,000 mol wt peptide–sodium dodecyl sulfate complex, its negative charge would be much less. Uricase molecules not dissociated or denatured in sodium dodecyl sulfate would have bound only a small fraction of the potential number of dodecyl sulfate ions and, thus, would have migrated more slowly in an electric field.

Uricase exhibited an even more striking resistance to dissociation and denaturation by 6 M guanidinium chloride. Only partial dissociation of the enzyme occurred in 6 M guanidinium chloride after 5 days at 25°. Elevated temperatures for short periods enhanced the dissociation, but total dissociation was not achieved (Figure 3). Disulfide bonds apparently made no contribution to structure stabilization since the presence of reducing agent had no effect in sodium dodecyl sulfate or guanidinium chloride.

We believe that we have obtained an accurate estimate of the molecular weight of the constituent polypeptide chains of uricase, although the data from each method are not unequivocal when considered alone. Sedimentation equilibrium in 6 M guanidinium chloride yields a mass for the subunits of 32,000. Only if all noncovalent interactions in the protein were ruptured and the subunits behaved as linear polymers, each of about 290 amino acids, could a mol wt of 32,000 be obtained by gel filtration in 6 M guanidinium chloride. Since this was the only size polypeptide chain observed by the latter method, it must be concluded that when disruption–denaturation of the protein occurred, all noncovalent interactions were broken, and the ultimate polypeptide chains were realized.

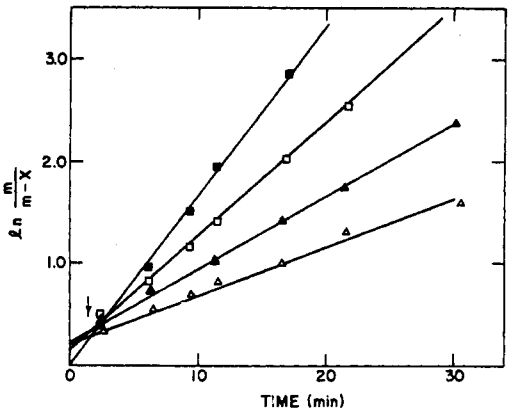

FIGURE 6: First-order reversible two-state model replot of early thermal inactivation data shown in Figure 5. According to the notation of Moore (1963), the integrated form of the rate law for a reversible, two-state model

$$A \underset{k_b}{\overset{k_f}{\rightleftharpoons}} B$$

may be expressed by

$$\ln [m/(m - x)] = (k_f + k_b)t$$

where $m = k_f a_0/(k_f + k_b)$. For this case a_0 is the initial concentration of A, and the initial concentration of B is zero. The apparent constant activity position approached in Figure 5 was estimated and used as a quasiequilibrium constant for preparation of this plot.

The action of sodium dodecyl sulfate upon protein structure and the final product of this action are entirely different from that of guanidinium chloride. Yet, the smallest polypeptide chain obtained, if complexed with sodium dodecyl sulfate to the usual extent, was one of about 290 amino acid residues or 32,000 mol wt. This is in total agreement with the results in guanidinium chloride. Uricase must then be composed of four chains of approximately equal mass.

A possible contributing factor to the resistance by uricase to guanidinium chloride denaturation is pointed out in Figure 4. The "competitive" inhibition pattern in reciprocal plots suggests that guanidinium ion is an inhibitor which binds to the same site as urate. Since the structural similarity between guanidinium ion and urate is not striking, it is reasonable that guanidinium ion is not an extremely potent inhibitor of uricase. However, the concentration of guanidinium chloride used for denaturation is high enough to saturate the active sites available and thus produce some degree of "substrate" protection to the enzyme.

Additional information with regard to the behavior of uricase toward denaturation was gained by using temperature as a perturbant. By measuring the uricase reaction rate as a function of temperature Mahler *et al.* (1955) observed significant deviation from linearity in Arrhenius plots above 40° We have generally confirmed this effect of temperature on the reaction rate and further investigated the direct effects of increased temperature on enzyme inactivation. The early part of these data could be fit to a reversible, first-order kinetic model (see Figure 6). It should be clearly pointed out that the two-state reversible model and the constants derived therefrom (see Table I) must be considered as descriptive approximations only, since we were unable to observe a reactivation using various slow cooling programs. The lack of attainment of true equilibrium is further evidenced by the continued slow decrease in activity following this initial period. The kinetic plots (Figures 5 and 6) show a nonzero intercept which can only partially be explained by error introduced through the time lag required to raise the enzyme to the desired temperature. This behavior becomes more pronounced at higher temperatures. Thus, it is possible that a rapid transition occurs prior to the predominant kinetic process observed (and replotted in Figure 6). Further studies will be required for verification.

In summary it appears that pig liver uricase is a globular metal-containing protein of native mol wt 125,000 and is composed of four equal sized subunits. The enzyme exhibits unusual resistance to denaturation by sodium dodecyl sulfate, guanidinium chloride, and thermal inactivation. The resistance to denaturation by guanidinium chloride can, in part, be explained by the direct interaction of the denaturant with the enzyme-active site.

Acknowledgments

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