Thermodynamics and Stoichiometry of the Binding of Substrate Analogues to Uricase

Thomas G. CONLEY and David G. PRIEST*

Department of Biochemistry, Medical University of South Carolina, 171 Ashley Avenue,
Charleston, SC 29403, U.S.A.

(Received 13 June 1979)

The subunit composition, metal content, substrate-analogue binding and thermal stability of *Aspergillus flavus* uricase were determined. *A. flavus* uricase is a tetramer and contains no copper, iron or any other common prosthetic group. Analytical-gel-filtration and equilibrium-dialysis experiments showed one binding site per subunit for urate analogues. The free energy of xanthine binding was -30.5 kJ (-7.3 kcal)/mol of subunit by equilibrium dialysis and -30.1 kJ (-7.2 kcal)/mol of subunit by microcalorimetry. The enthalpy change for xanthine binding was -15.9 kJ (-3.8 kcal)/mol of subunit when determined from the temperature-dependence of the equilibrium constant and -18.0 kJ (-4.3 kcal)/mol of subunit when measured microcalorimetrically. The thermal inactivation rate of *A. flavus* uricase increases as protein concentration is decreased. This concentration-dependent instability is not due to subunit dissociation.

Uricase (urate–oxygen oxidoreductase, EC 1.7.3.3) from pig liver consists of four apparently identical subunits (Pitts *et al.*, 1974). However, because of the reported metal content of 1 mol of copper/mol of enzyme (Mahler *et al.*, 1955), a single catalytic site has been assumed (Pitts & Priest, 1974). There is now some question about the presence of functional copper in uricase from several other sources (Roush & Shieh, 1963; Nose & Arima, 1968; Itaya *et al.*, 1971; Kosman, 1978).

The results of studies of the binding of substrate analogues obtained with uricase from *Aspergillus flavus* and pig liver have now led to an unambiguous determination of the binding stoichiometry. Thermodynamic parameters for substrate-analogue binding and thermal-stability properties are also reported.

Materials and Methods

A. flavus uricase was obtained from Boehringer Mannheim Corp. (New York, NY, U.S.A.) as a freeze-dried powder. The enzyme was dissolved in 0.1 M-glycine/NaOH buffer, pH 8.8, and dialysed extensively against this buffer before use. Pig uricase was purified as previously described (Conley & Priest, 1979). Uricase activity was determined by monitoring the decrease in urate absorbance at

290 nm by using a Beckman ACTA C-III recording spectrophotometer with thermostatically controlled cell chamber. Uric acid was obtained from Calbiochem (Los Angeles, CA, U.S.A.), and solutions were prepared fresh daily. Assays were performed at 24°C unless otherwise stated. Assay mixtures consisted of 3.0 ml of 0.1 M-potassium borate buffer, pH 8.8, containing $100 \mu\text{M}$ -urate at atmospheric O_2 partial pressure. A molar absorption coefficient of $1.23 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for urate was used (Kalckar, 1947). The concentration of *A. flavus* uricase was determined by using an absorption coefficient ($A_{281}^{1\%}$) of 16.9. The concentration of pig uricase was determined by using an $A_{276}^{1\%}$ value of 11.3 (Mahler, 1963), after this value had been confirmed.

The copper and iron contents of *A. flavus* uricase were determined by compleximetric techniques. Chelating reagents were obtained from Calbiochem. Cuproin (Oehlmann, 1957; Riley & Sinhaseni, 1958), neocuproin (Sherwood & Chapman, 1955; Zall *et al.*, 1957) and bathocuproin (Borchardt & Butler, 1957; Smith & Wilkins, 1953) were used for the detection of copper. *o*-Phenanthroline (Feigl & Caldas, 1957; Peters *et al.*, 1956) and bathophenanthroline (Kingsley & Getchell, 1956; Seven & Peterson, 1958) were used for the detection of iron. Typically, 4 mg of uricase was dialysed against three 4-litre volumes of deionized water, then digested with conc. H_2SO_4 . Samples for iron determination were treated with 70% (v/v) HClO_4 ; samples for copper

* To whom request for reprints should be addressed.

determination were treated with 20% (v/v) hydroxylamine. The samples were extracted with isopentanol containing the appropriate chelating agent, and the concentrations of the metal complexes were determined spectrophotometrically.

The molecular weight and the absolute protein concentration of *A. flavus* uricase were determined by sedimentation-equilibrium experiments at 25°C in a Beckman model E analytical ultracentrifuge. The solvent employed was 0.1 M-glycine/NaOH buffer, pH 8.8. Experimentally determined values of 1.0024 gm/cm³ and 1.0203 at 25°C for solvent density and relative viscosity respectively were used. The molecular weight was determined by the short-column meniscus-depletion method of Yphantis (1964). Absolute protein concentration was determined by the method of Babul & Stellwagen (1969).

Gel chromatography was performed on a 2.5 cm × 70 cm jacketed column of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.). The column was equilibrated with 0.1 M-glycine/NaOH buffer, pH 8.8, and calibrated by the method of Andrews (1964). Zonal gel filtration (Ackers, 1967; Chiancone *et al.*, 1968) was performed on the same column at 24°C and at 45°C. Typically 20 ml of solution containing from 0.002 to 1.0 mg of protein/ml was pumped on to the column and eluted at a rate of 5 ml/min. The effluent was monitored at 280 nm. Measurement of the number of binding sites by the gel-filtration method of Hummel & Dreyer (1962) was performed with a 2.5 cm × 70 cm jacketed column of Sephadex G-25 at 24°C with saturating concentrations (at least $10^2 \times K_f$) of ligand. Experiments with pig uricase were performed in 0.1 M-sodium carbonate buffer, pH 10.2, and those with *A. flavus* uricase in 0.1 M-glycine/NaOH buffer, pH 8.8.

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed according to the method of Weber & Osborn (1969).

Equilibrium-dialysis experiments were performed with a multichamber equilibrium-dialysis cell (Chemical Rubber Co.). [¹⁴C]Xanthine was obtained from ICN (Irvine, CA, U.S.A.) (specific radioactivity 57 mCi/mmol). A 0.5 ml sample of 0.1 M-glycine/NaOH buffer, pH 8.8, containing from 0.5 to 2.0 mg of uricase was placed on one side of the dialysis membrane, and 0.5 ml of [¹⁴C]xanthine solution was placed on the other. Samples were equilibrated for 18 h at the desired temperature in a shaking water bath. Portions (50 μl) were withdrawn, diluted into 15 ml of Bray's (1960) solution and their radioactivities counted in a Packard Tri-Carb liquid-scintillation spectrometer.

Microcalorimetric experiments were performed with an LKB batch microcalorimeter equipped with gold cells. Experiments were performed at 24°C.

Typically, 2 ml of 0.1 M-glycine/NaOH buffer, pH 8.8, containing 6 mg of uricase was placed in one side of the reaction cell and 4 ml of xanthine solution was placed in the other. Exact amounts added were determined by weighing the syringes used to transfer the solutions before and after transfer. The cell compartment was sealed and allowed to come to thermal equilibrium. The ligand and enzyme were mixed, and the heat of binding was measured. The calorimeter was calibrated electrically. The heat of dilution of sucrose (Gucker *et al.*, 1939) was measured periodically as a check of calorimeter stability.

Results

Table 1 shows that neither copper nor iron was detected in significant quantities in fully active *A. flavus* uricase. Three different, and relatively specific, chelating agents were used for the detection of copper, and two for the detection of iron. Less than 0.1 mol of either metal/mol of enzyme was detected, and copper or iron, added at a ratio of 0.5 mol of metal/mol of uricase, could be recovered.

Pig liver uricase has been shown to be a tetramer (Pitts *et al.*, 1974). A copper content of 1 mol of copper/mol of uricase (Mahler *et al.*, 1955) has led to the assumption of a single substrate-binding site (Pitts & Priest, 1974). Table 2 shows that the molecular weights, shapes and subunit compositions of *A. flavus* and pig uricases are very similar. A slight difference occurs in the spectral properties of the two enzymes, and there is a pronounced difference in solubility characteristics.

Even if O₂ is nearly completely removed from reaction mixtures by N₂ bubbling, some turnover can still occur, making interpretation of urate-binding data difficult. Therefore the unreactive urate analogues xanthine and trichloropurine were used to

Table 1. *Absence of copper and iron from A. flavus uricase*

Acid digests of the proteins were extracted with isopentanol containing the chelating agents cuproin, neocuproin and bathocuproin for copper, and *o*-phenanthroline and bathophenanthroline for iron. Concentrations of the metal complexes were determined spectrophotometrically. Samples (1 ml) containing 4.0 mg of uricase (32 μM) were used in each experiment. Values shown reflect the number of mol of metal bound/mol of enzyme.

Metal	Added metal (mol/mol of uricase)	Recovered metal (mol/mol of uricase)
Copper	None	≤0.06
Copper	0.50	0.46 ± 0.05
Iron	None	≤0.08
Iron	0.50	0.58 ± 0.03

Table 2. *Physical properties of pig and A. flavus uricases*
The specific activity of the *A. flavus* uricase was 16.9 units ($\mu\text{mol/min}$)/mg.

Property	Pig uricase	<i>A. flavus</i> uricase
Molecular weight	125 000*	130 000§
$s_{20,w}^0$ (S)	6.85*	6.75
f/f_{min}	1.34*	1.29§
Subunit molecular weight	32 000*	32 000¶
λ_{max} (nm)	276†	281
$A_{\text{max}}^{1\%}$	11.3†	16.9**
Solubility (mg/ml)		
pH 10.0‡	<5	>10
pH 7.0‡	<0.05	>10

* Values are those reported by Pitts *et al.* (1974).

† Values are those reported by Mahler (1963).

‡ Buffers used were 0.1 M-sodium carbonate at pH 10.0 and 0.1 M-potassium phosphate at pH 7.0. The enzymes were dissolved by vortex-mixing in the appropriate buffer for 2 min, centrifuged to remove undissolved protein, and the dissolved protein concentration was determined by measuring the absorbance at the appropriate λ_{max} .

§ Determined from sedimentation-equilibrium data.

|| Determined from sedimentation-velocity data.

¶ Determined from relative mobility on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate.

** The absolute protein concentration was calculated by the method of Babul & Stellwagen (1969).

investigate the number of purine-binding sites on uricase. Both of these compounds behave as classical competitive inhibitors of uricase with respect to urate (xanthine, $K_1 = 3.0 \mu\text{M}$; trichloropurine, $K_1 = 0.4 \mu\text{M}$). Gel-filtration experiments, performed by the method of Hummel & Dreyer (1962), were used to estimate an *A. flavus* uricase binding stoichiometry of 3.8 ± 0.4 for xanthine and 4.1 ± 0.3 for trichloropurine. The relative insolubility of pig uricase prevented precise stoichiometry estimates, but both trichloropurine and xanthine bound at a ratio significantly greater than 1:1.

Fig. 1 shows that inactivation occurred when *A. flavus* uricase samples were maintained at elevated temperatures. Similar inactivation profiles obtained with pig uricase have been shown to fit a reversible first-order two-state (fully active and totally inactive enzyme) kinetic model (Pitts *et al.*, 1974). Such a model can also be used for *A. flavus* uricase. Fig. 2 shows that the thermal inactivation of *A. flavus* uricase is also enzyme-concentration-dependent. Rate constants for both the forward and the reverse processes are shown in Table 3. The insolubility of pig uricase made such studies difficult with that enzyme.

Fig. 1. Decrease in *A. flavus* uricase initial velocity with time at elevated temperatures

Samples (1 ml) containing 0.010 mg of uricase/ml in 0.1 M-glycine/NaOH buffer, pH 8.8, were immersed in a water bath at 24°C (○), 35°C (□), 45°C (△), 51°C (◐) or 63°C (●). After 1.5 min, for temperature equilibration, samples were withdrawn and initial rates were determined at 290 nm in 0.1 M-sodium borate buffer, pH 8.8, containing 100 μM -urate at 24°C. A value of 1 on the relative initial-velocity scale corresponds to a specific activity of 16.9 units/mg.

Fig. 2. Enzyme-concentration-dependence of the decrease in *A. flavus* uricase initial velocity with time at 45°C. Concentrations used were 0.91 mg/ml (○), 0.106 mg/ml (□), 0.053 mg/ml (△) and 0.010 mg/ml (●). Other conditions are the same as in Fig. 1.

Fig. 3 shows that subunit dissociation is not the source of the concentration-dependent thermal instability of uricase. Zonal analytical-gel-filtration experiments were conducted over a protein con-

Table 3. Apparent first-order rate constants for thermal inactivation of *A. flavus* uricase

Rate constants were calculated from the results shown in Figs. 1 and 2 by the method previously reported (Pitts *et al.*, 1974).

Temperature (°C)	Uricase (mg/ml)	k_f (min ⁻¹)	k_b (min ⁻¹)
45	0.053	0.055	0.096
45	0.010	0.084	0.067
51	0.010	0.077	0.017

Fig. 3. Zonal gel filtration of *A. flavus* uricase. A 2.5 cm $\times$ 70 cm column of Sephadex G-200, equilibrated with 0.1 M-glycine/NaOH buffer, pH 8.8, was used at 24°C (O) and at 45°C ($\blacktriangle$). Typically 20 ml of protein solution was pumped on to the column and eluted at a flow rate of 5 ml/h. The elution volume ($\bar{V}$) is defined as the centroid of the leading edge of the protein zone (Ackers, 1967).

Fig. 4. Equilibrium-dialysis measurements of xanthine binding to *A. flavus* uricase

A sample (2 mg) of uricase in 0.5 ml of 0.1 M-glycine/NaOH buffer, pH 8.8, was placed on one side of a membrane, and xanthine in the same buffer system was placed on the other. After 18 h the concentration of [¹⁴C]xanthine was determined on each side of the membrane, and the amount bound was calculated. Results obtained at 16°C ($\bullet$), 24°C (Δ) and at 35°C (O) are plotted in accordance with the Scatchard (1949) relationship.

Fig. 5. Temperature-dependence of the *A. flavus* uricase-xanthine binding constant

Conditions were the same as described in Fig. 4. Points represent the averages of at least five separate determinations and error bars the standard deviation.

Fig. 6. Reciprocal plot of microcalorimetrically determined heat associated with xanthine binding to *A. flavus* uricase

The heat of dilution of the xanthine solution was blanked out in each experiment; the heat of dilution of the enzyme was measured separately and the binding heats were corrected accordingly. As recommended by Bolen *et al.* (1971), $Q_{\max}$ was measured at high ligand concentration and used to calculate the concentration of free xanthine. Binding was performed at atmospheric O₂ partial pressure (O) and at 100% N₂ (Δ).

centration range that exhibited inactivation. No change was observed in the elution volume ($\bar{V}$) of the leading edge of the protein band. Results were identical at 24°C and 45°C.

Table 4. *Thermodynamic parameters for the binding of xanthine to A. flavus uricase at 24°C*

Values of ΔG^0 , ΔH^0 and ΔS^0 are given per mol of subunit. Free-energy change was calculated from the relationship $\Delta G^0 = -RT \cdot \ln K_b$, and entropy change was calculated from the relationship $\Delta G^0 = \Delta H^0 - T\Delta S^0$.

	K_b (M^{-1})	ΔG^0 (kJ/mol)	ΔH^0 (kJ/mol)	ΔS^0 ($J \cdot mol^{-1} \cdot K^{-1}$)
Equilibrium dialysis	2.33×10^5	-30.5	-15.9	49.4
Microcalorimetry	2.11×10^5	-30.1	-18.0	41.0

Equilibrium dialysis was used to measure xanthine binding. Linear Scatchard (1949) plots were obtained at each temperature used (see Fig. 4). A binding constant of $2.33 \times 10^5 M^{-1}$ was estimated at 24°C. Identical results were obtained over the enzyme concentration range 0.5–2.0 mg/ml.

Binding constants were determined as a function of temperature over the range 16–35°C. When they were plotted according to the van't Hoff relationship (see Fig. 5), an enthalpy change of -15.9 kJ (-3.8 kcal)/mol of binding sites was obtained. Microcalorimetry was also used to measure the enthalpy change for xanthine binding at 24°C (see Fig. 6). Extrapolation to infinite xanthine concentration allowed an estimate of -18.0 kJ (-4.3 kcal)/mol of binding sites. Essentially identical results were obtained in 0.1 M-glycine and 0.1 M-Tris buffer systems at pH 8.8. Since these two buffers have different heats of ionization (Hinz *et al.*, 1971), proton dissociation does not appear to accompany xanthine binding. A summary of the thermodynamic parameters for xanthine binding, including the entropy change at 24°C, is shown in Table 4.

Discussion

Uricase from *A. flavus* and pig liver are both tetrameric. The *A. flavus* enzyme contains one purine-binding site per subunit. The relative insolubility of the pig enzyme made stoicheiometry estimates less precise. Nevertheless urate analogues bound at a ratio clearly greater than 1 molecule per tetramer. Therefore previous assumptions of substrate-binding stoicheiometry, based on apparent copper content (Pitts & Priest, 1974), are incorrect. Since copper is not present in *A. flavus* uricase, and if present in the pig enzyme is not required for purine binding, it is unlikely that copper plays a direct role in enzymic catalysis of urate oxidation.

Both the *A. flavus* and pig enzymes are thermally inactivated in a manner that fits a reversible first-order kinetic model. The *A. flavus* enzyme also exhibits a protein-concentration-dependence for this thermal inactivation. Subunit dissociation was ruled out as the cause of the concentration-dependent lability of the *A. flavus* enzyme. Dissociation of a prosthetic group could account for the concentration-dependence, but the present evidence sug-

gests that the enzyme does not contain a readily dissociable cofactor.

Below 35°C the enzyme was sufficiently stable for us to conduct equilibrium binding studies with urate analogues. Close inspection of binding isotherms can be used to test for interaction between binding sites. With the use of linearized plotting forms no evidence could be obtained for such interactions by equilibrium dialysis or microcalorimetric measurements. Both techniques yielded essentially the same binding constant at 24°C. This constant compares quite well with the steady-state kinetic inhibition constant, K_i (3.0 μM), for xanthine. For comparison, the binding constant in Table 4 can be expressed as a dissociation constant K_d (4.3 μM).

The enthalpy change for xanthine binding to *A. flavus* uricase, as determined from the temperature-dependence of the binding constant and by microcalorimetry, are in very close agreement. No protein dissociation could be detected from the microcalorimetric measurements, nor would such close agreement be expected between the two techniques if such were the case. The relatively small enthalpy change when combined with the equilibrium constant at 24°C yields a positive entropy change. Neither the enthalpy term nor the entropy term dominate the free-energy equation at 24°C. Such an entropy change is consistent with a decrease in the degree of hydration on binding of xanthine.

We express our appreciation to Dr. W. W. Fish and Mr. James Collawn for their help with the ultracentrifuge experiments. Financial assistance was provided by a grant from the Research Corporation.

References

- Ackers, G. K. (1967) *J. Biol. Chem.* **242**, 3026–3034
- Andrews, P. (1964) *Biochem. J.* **91**, 222–223
- Babul, J. & Stellwagen, E. (1969) *Anal. Biochem.* **28**, 216–221
- Bolen, D. W., Flogel, M. & Biltonen, R. (1971) *Biochemistry* **10**, 4136–4140
- Borchardt, L. G. & Butler, J. P. (1957) *Anal. Chem.* **29**, 414–419.
- Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
- Chiancone, E., Gilbert, L. M., Gilbert, G. A. & Kellett, G. L. (1968) *J. Biol. Chem.* **243**, 1212–1219
- Conley, T. G. & Priest, D. G. (1979) *Prep. Biochem.* **9**, 197–203

- Feigl, F. & Caldas, A. (1957) *Anal. Chem.* **29**, 580–585
- Gucker, F. T., Pickard, H. B. & Planck, R. W. (1939) *J. Am. Chem. Soc.* **61**, 459–464
- Hinz, H. J., Shaio, D. D. F. & Sturtevant, J. M. (1971) *Biochemistry* **10**, 1347–1353
- Hummel, J. P. & Dreyer, W. J. (1962) *Biochim. Biophys. Acta* **63**, 530–532
- Itaya, K., Fukumoto, J. & Yamamoto, T. (1971) *Agric. Biol. Chem.* **35**, 813–821
- Kalckar, H. M. (1947) *J. Biol. Chem.* **167**, 429–443
- Kingsley, G. R. & Getchell, G. (1956) *Clin. Chem.* **2**, 175–183
- Kosman, D. J. (1978) in *Bioorganic Chemistry* (Van Tamelen, E. E., ed.), vol. 2, pp. 175–195, Academic Press, New York
- Mahler, H. R. (1963) *Enzymes 2nd Ed.* **8**, 285–296
- Mahler, H. R., Hübscher, G. & Baum, H. (1955) *J. Biol. Chem.* **216**, 625–641
- Nose, K. & Arima, K. (1968) *Biochim. Biophys. Acta* **151**, 63–69
- Ochlmann, F. (1957) *Chem. Technol.* **9**, 599–601
- Peters, T., Giovanniello, T. J., Apt, L., Ross, J. F. & Trakas, A. P. (1956) *J. Lab. Clin. Med.* **48**, 280–288
- Pitts, O. M. & Priest, D. G. (1974) *Arch. Biochem. Biophys.* **163**, 359–366
- Pitts, O. M., Priest, D. G. & Fish, W. W. (1974) *Biochemistry* **13**, 888–892
- Riley, J. P. & Sinhaseni, P. (1958) *Analyst (London)* **83**, 299–302
- Roush, A. H. & Shieh, T. R. (1963) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **22**, 292
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Seven, M. J. & Peterson, R. E. (1958) *Anal. Chem.* **30**, 2016–2018
- Sherwood, R. M. & Chapman, F. W. (1955) *Anal. Chem.* **27**, 88–93
- Smith, G. F. & Wilkins, D. H. (1953) *Anal. Chem.* **25**, 510–585
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Yphantis, D. A. (1964) *Biochemistry* **3**, 297–317
- Zall, D. M., McMichael, R. E. & Fischer, D. W. (1957) *Anal. Chem.* **29**, 88–92