Note

Reversible Inactivation of an Intracellular Uricase from *Bacillus fastidiosus* via Dissociation of Homotetramer into Homodimers in Solutions of Low Ionic Strength

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An intracellular uricase from *Bacillus fastidiosus* with high catalytic capacity and strong resistance to xanthine was inactivated in water but could be essentially re-activated in solutions of high ionic strength. By polyacrylamide gel electrophoresis (PAGE), gradient PAGE, sodium-dodecyl-sulfate-PAGE, gel-filtration through Sephadex G200, and activity staining with peroxidase against water to remove organic contaminants that have potential interference with subsequent modification of proteins by desired polymers, but it could be essentially re-activated in solutions of high ionic strength. Sensitivity to low ionic strength of solutions complicates formulation of this uricase as a drug and its elimination requires protein engineering.

**Key words:** uricase; dialysis; inactivation; dissociation of homotetramer; ionic strength

In many invertebrates, plants, and micro-organisms, uric acid is converted to allantoin by uricase and consecutive enzymes.1) Human body lacks uricase activity and uric acid as the end-product during degradation of purine bases is passed out through kidneys. Elevated levels of uric acid in the plasma, i.e., hyperuricemia, are associated with many pathological processes, including gout, kidney function disorders, and cardiovascular diseases.2) Hence, serum uric acid is routinely monitored in clinical laboratories and uricase is widely used as a key tool in enzymatic analyses of serum uric acid.3,4) Moreover, there is tumor lysis syndrome during chemotherapy of cancers such as leukemia and lymphoma, which causes hyperuricemia and acute kidney dysfunction.5–7) And for some patients of severe gout, xanthine oxidase inhibitors like allopurinol provide no improvement.8) In these cases, uricases are more powerful to prevent and treat hyperuricemia.8–11) Furthermore, xanthine as the precursor of uric acid is a potent competitive inhibitor of common uricases,3,12) and hyperuricemia is usually associated with hyperxanthinemia.13) Therefore, uricases with high catalytic capacity and resistance to xanthine are highly valuable in biomedicine.

Recently, we discovered an intracellular uricase from *Bacillus fastidiosus* A.T.C.C 26904 with superior catalytic capacity, and the inhibition constant of xanthine on it was about 6 times those on common fungal uricases.12) To treat hyperuricemia, uricase must be modified with polymers like polyethylene glycol to block its immunoreactivity.14,15) Unexpectedly, this intracellular uricase from *Bacillus fastidiosus* was inactivated during dialysis against water to remove organic contaminants that have potential interference with subsequent modification of proteins by desired polymers, but it could be essentially re-activated in solutions of high ionic strength. This unusual property complicates its formulation as a therapeutic protein drug. Here, we investigated a possible mechanism for reversible inactivation of this intracellular uricase in solutions of low ionic strength.

This intracellular uricase was prepared to above 90% homogeneity by anion-exchange chromatography with 50.0 mM Tris–HCl buffer at pH 8.0 and preparative polyacrylamide gel electrophoresis (PAGE).15) To measure initial rates at 25 °C, uric acid (Alfa Aesar, Tianjing, China) was added to sodium borate buffer (100.0 mM, pH 9.2, containing 0.10 mM diethylenetriaminepenta-acetic acid, which was used throughout unless otherwise stated) to a final 75.0 μM to initiate the uricase reaction, and one unit of uricase was the amount that was able to oxidize one micromole uric acid per min.12,16) The Bradford method was used to quantify protein concentration, with bovine serum albumin (BSA) as a standard.17)

This intracellular uricase showed unchanged specific activities after dialysis for 24 h at 4 °C against 50.0 mM Tris–HCl buffer at pH 8.0. However, after dialysis for 24 h at 4 °C against 1.0 mM Tris–HCl buffer at pH 8.0, residual specific activity of this uricase was below 10% of that stored in 50.0 mM Tris–HCl buffer at pH 8.0, and after dialysis for 24 h at 4 °C against distilled water, the residual specific activity was even below 5% of that stored in 50.0 mM Tris–HCl buffer at pH 8.0. The concentration of Tris–HCl buffer at pH 8.0 must not be less than 40.0 mM to maintain activity of this uricase over 24 h. Moreover, this uricase inactivated by dialysis could be partially re-activated in solutions of high ionic strength. To accelerate re-activation using amounts of

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Abbreviations: ADH, alcohol dehydrogenase; BSA, bovine serum albumin; OVA, ovalbumin; PAGE, polyacrylamide gel electrophoresis
this uricase as small as possible, this uricase after being inactivated by dialysis against water was directly incubated in the indicated solutions rather than being dialyzed against the indicated solutions. After incubation for 2 h in a solution of 100.0 mM sodium chloride in water at 4 °C, the inactivated uricase showed about 70% of the maximal specific activity. After incubation for 2 h in 100.0 mM Tris–HCl buffer at pH 8.0 plus 100.0 mM NaCl at 4 °C, the inactivated uricase showed about 90% of the maximal specific activity. After pre-incubation for 0.5 h in the sodium borate buffer plus 100.0 mM NaCl at 25 °C followed by the direct addition of uric acid to measure its activity, the inactivated uricase showed about 80% of the maximal specific activity. These results support that this uricase was reversibly inactivated in solutions of low ionic strength.

This uricase was sensitive to trypsin (data not shown). To check possible degradation of polypeptides during dialysis against water, the effects of common antibacterial agents, including sodium azide and penicillin, were tested. At levels that had no effects on uricase but were able to block growth of sensitive bacteria, none of the tested antibacterial agents prevented the inactivation of this uricase during dialysis against water. And common protease inhibitors, including 2.0 mM p-aminobenzamidine, 1.0 mM p-methylbenzoysulfonlfuryl fluoride, and 2.0 mM ethylenediamine tetraacetic acid, did not prevent the inactivation of this uricase during dialysis against water yet. Moreover, no protein bands for degraded subunits of this uricase after dialysis against water followed by partial re-activation were detected by sodium dodecyl sulfate-PAGE (SDS–PAGE, Fig. 1a).

These results support that the inactivation of this uricase in solutions of low ionic strength was independent of hydrolyses of polypeptides.

Reversible re-activation of this uricase confirmed no involvement of diffusible cofactors. This native intracellular uricase from Bacillus fastidiosus was a homotetramer of 144 kD. To investigate possible dissociation of active homotetramer of this uricase during dialysis against water, non-denaturing PAGE analysis of this uricase was performed. By non-denaturing PAGE, only one protein band was detected in this uricase showing full activity whereas an additional protein band moving at a rate comparable to that of BSA was detected in the uricase inactivated by dialysis against water. With decreasing activities of this uricase after dialysis against water followed by partial re-activation in solutions of high ionic strength, abundance of the band detected by non-denaturing PAGE for the native uricase continuously decreased whereas that of the additional band detected only for the inactivated uricase continuously increased (Fig. 1b). These results support that this uricase possibly dissociated into inactive oligomers, or even monomers, in solutions of low ionic strength.

To reveal possible composition of inactive oligomers dissociated from homotetramer of this uricase during
dialysis against solutions of low ionic strength, non-denaturing PAGE with continuous gradient of acrylamide from 4% to 30% was performed. This intracellular uricase had an isoelectric point around 5.0 (GeneBank, accession no. FJ393559), close to those of ovalbumin (OVA) and BSA. In addition, homodimer of this uricase had molecular weight comparable to that of BSA, suggesting that homodimer of this uricase should move at a rate comparable to that of BSA during gradient PAGE. The native uricase showing full activity gave one unique protein band during gradient PAGE, but the uricase inactivated by dialysis against water gave two bands, with an additional band moving at a rate faster than that of the native uricase but very close to that of BSA (Fig. 1c). A monomer of subunit of this uricase should move at a rate slightly faster than that of OVA, but this band was not detected after this uricase was inactivated by dialysis against water. These results support that this uricase dissociated mainly into inactive homodimers in solutions of low ionic strength.

To verify activities of the two protein bands resolved by non-denaturing PAGE in this uricase inactivated by dialysis against water, activities of the resolved protein bands were stained with uric acid, horseradish peroxidase, and diaminobenzidine tetrahydrochloride (both reagents were from Sangon Biotechnology, Shanghai, China). After non-denaturing PAGE, the gel was recovered immediately and rinsed with 10% glycerol in sodium borate buffer. Ten min later, the gel was transferred to the sodium borate buffer containing 200 U·1⁻¹ peroxidase with gentle shaking for a further 10 min. Finally, uric acid powder (final 0.4 mM) and a tablet of diaminobenzidine tetrahydrochloride were added to this buffered solution, and the gel was soaked in the solution at 25 °C with gentle shaking. Within 10 min, the deposit of brown chromatogen was eyesight-visible with the native uricase, which grew dark brown in 30 min. Nevertheless, after staining for 25 min, there was also a visible deposit of slightly brown chromatogen with the inactivated uricase at the position close to that of BSA (Fig. 1d), which might have been due to the reversible formation of active homotetramers during staining. Therefore, reversible inactivation of this uricase during dialysis against water was due to reversible dissociation of its active homotetramer into inactive homodimers in solutions of low ionic strength, and electrostatic repulsion might have played a role in its dissociation.

To confirm the formation of inactive homodimers of this uricase in solutions of low ionic strength, gel filtration (Sephadex G200, Amersham Biosciences, Chalfont St. Giles, UK) was further performed with blue dextran, yeast alcohol dehydrogenase (ADH, Sigma-Aldrich, St. Louis, MO, USA) and BSA as references (Fig. 2a). Elution was monitored by absorbance at 280 nm and the activity of uricase or ADH. For the native uricase (activity about 11.0 U·mg⁻¹) eluted with 100.0 mM Tris–HCl buffer at pH 8.0, monitoring absorbance at 280 nm gave a unique peak that had an elution volume consistent with that of ADH, and monitoring uricase activity gave exactly the same unique peak regardless of pre-incubation in the sodium borate buffer plus 100.0 mM NaCl (Fig. 2b). The elution of blue dextran or BSA with 1.0 mM Tris–HCl buffer at pH 8.0 from the same column gave a peak position consistent with that eluted with 100.0 mM Tris–HCl buffer at pH 8.0. For this uricase after dialysis against 1.0 mM Tris–HCl at pH 8.0 for 8 h to exhibit about 20% of the maximal specific activity, there were two peaks by absorbance at 280 nm during elution with 1.0 mM Tris–HCl at pH 8.0. In this case, one peak had an elution volume close to that of BSA but showed negligible uricase activity whereas the other had an elution volume comparable to that of BSA (Fig. 1d), which might have been due to the reversible formation of active homotetramers during dialysis against 1.0 mM Tris–HCl buffer at pH 8.0. Absorbance at 280 nm was monitored (→). Every 0.90 ml eluent was collected to measure ADH activity with 50 μl eluent in a 1.20 ml reaction mixture (▲▲, U⁻¹). One unit of ADH was that to produce one micromole NADH per min. b, Fully active uricase at 11.0 U·mg⁻¹, 2.0 mg in total in 1.20 ml Tris–HCl buffer (100.0 mM, pH 8.0), were loaded and eluted with the equilibrating buffer at 0.18 ml·min⁻¹. Absorbance at 280 nm was monitored (→). Every 0.90 ml eluent was collected. Uracase activity (○○, U⁻¹) was measured with 50 μl eluent in a 1.20 ml mixture of the sodium borate buffer. Incubation of 50 μl eluent for 0.5 h at 25 °C in the sodium borate buffer plus 100.0 mM NaCl gave consistent uricase activity. c, The same column was equilibrated with 1.0 mM Tris–HCl buffer at pH 8.0 and calibrated with Blue dextran and BSA as described above. Then, 2.0 mg uricase in 1.20 ml Tris–HCl buffer (1.0 mM, pH 8.0) with total activity of 4.8 U, after dialysis for 8 h at 4 °C against 1.0 mM Tris–HCl buffer at pH 8.0, was loaded and eluted at 0.18 ml·min⁻¹ with 1.0 mM Tris–HCl buffer at pH 8.0. Absorbance at 280 nm was monitored (→). Every 0.90 ml eluent was collected. Uracase activity (U⁻¹) was directly measured with 50 μl eluent in a 1.20 ml mixture of the sodium borate buffer (××) or after pre-incubation of 50 μl eluent for 0.5 h at 25 °C in the sodium borate buffer plus 100.0 mM NaCl (○○), indicated consistent uricase activity after pre-incubation of eluent in the sodium borate buffer plus 100.0 mM NaCl.
consistent with that of ADH and showed obvious uricase activity. Nevertheless, the peak with the elution volume close to that of BSA showed uricase activity after pre-incubation in the sodium borate buffer plus 100.0 mM NaCl for 0.5 h at 25°C (Fig. 2c). These results confirm that this uricase dissociated into inactive homodimers during dialysis against solutions of low ionic strength.

Sensitivity of quaternary structures to stress conditions is a common property of proteins,19–21 but such high sensitivity of tetrameric structure of this uricase to ionic strength of solutions was really rare. Recombinant expression of eukaryotic uricases usually tolerate higher cost.22 This uricase from Bacillus fastidious reserved more than 45% of the maximal activity after modification with monomethoxyl-poly(ethylene glycol) ester (data not shown), confirming that it was a candidate drug to treat hyperuricemia-associated diseases. Coding sequence of this uricase is available (GeneBank, accession no. FJ393559), and protein engineering to eliminate its sensitivity to dialysis against water is currently underway to improve its potential use as a therapeutic enzyme.

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References