The structure of urease activation complexes examined by flexibility analysis, mutagenesis, and small-angle X-ray scattering

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Conformational changes of Klebsiella aerogenes urease apoprotein (UreABC) induced upon binding of the UreB and UreF accessory proteins were examined by a combination of flexibility analysis, mutagenesis, and small-angle X-ray scattering (SAXS). ProFlex analysis of urease provided evidence that the major domain of UreB can move in a hinge-like motion to account for prior chemical cross-linking results. Rigidification of the UreB hinge region, accomplished through a G11P mutation, reduced the extent of urease activation, in part by decreasing the nickel content of the mutant enzyme, and by sequestering a portion of the urease apoprotein in a novel activation complex that includes all of the accessory proteins. SAXS analyses of urease, (UreABC–UreD)\textsubscript{3}, and (UreABC–UreDF)\textsubscript{3} confirm that UreD and UreF bind near UreB at the periphery of the (UreAC)\textsubscript{3} structure. This study supports an activation model in which a domain-shifted UreB conformation in (UreABC–UreDF)\textsubscript{3} allows CO\textsubscript{2} and nickel ions to gain access to the nascent active site.

Urease is a nickel-containing enzyme that hydrolyzes urea [1,2]. Crystallographic analyses of ureases from bacterial and plant sources [3–7] reveal a basic trimeric structure with three active sites, each composed of two nickel ions coordinated by a carboxylated Lys, four His and an Asp. Genetic and biochemical studies carried out with plants, fungi, and bacteria [reviewed in [8–10]] have shown that additional genes encoding accessory proteins are required for proper assembly of the urease metallocenter, with the possible exception of that from Bacillus subtilis [11]. The current model for urease metallocenter assembly (Fig. 1) derives primarily from studies involving expression of the Klebsiella aerogenes ureD–ABCEFG gene cluster in Escherichia coli [reviewed in [8,12]]. The active enzyme possesses three copies of each of three subunits (UreA, UreB, and UreC of M\textsubscript{r} 11,086, 11,695, and 60,304, respectively) [13]. Deletions within ure\textsubscript{D}, ure\textsubscript{F}, ure\textsubscript{G} eliminate urease activity due to production of the inactive (UreABC)\textsubscript{3} ureaprotein [14]. Expression of ureDABC produces (UreABC–UreD)\textsubscript{3} with UreD (M\textsubscript{r} 29,300) in complex with urease apoprotein [15]. Co-expression of ure\textsubscript{F} (encoding a protein of M\textsubscript{r} 25,221) with ureDABC produces (UreABC–UreDF)\textsubscript{3} [16]. The soluble protein UreG (M\textsubscript{r} 21,943) reversibly binds to (UreABC–UreDF)\textsubscript{3}, forming (UreABC–UreDFG)\textsubscript{3} [17,18]. Urease activity is generated by incubating these complexes with high concentrations of bicarbonate (to supply the CO\textsubscript{2} needed for Lys carboxylation) and nickel ions, but the required levels of these additives (100 mM and 100 \mu M, respectively) are not physiologically relevant and only a portion of the proteins are activated [19,20]. In contrast, fully active urease is generated with only 100 \mu M bicarbonate and 20 \mu M nickel ions using (UreABC–UreDFG)\textsubscript{3} plus UreE (M\textsubscript{r} 17,558) and GTP [21]. UreE functions as a nickel-binding protein [22,23] that delivers the metal ion to (UreABC–UreDFG)\textsubscript{3} as GTP is hydrolyzed [24]. Although UreE is often referred to as a metallochaperone [25,26] and UreDFG has been termed a urease-specific molecular chaperone [9], the mechanism of urease metallocenter assembly has remained obscure.

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The program identifies the flexible and rigid regions in a given structure (which bonds are constrained and which bonds remain free to rotate) based on analysis of constraints posed by the protein’s network of covalent bonds, hydrogen bonds, salt bridges, and hydrophobic interactions [34]. ProFlex calculations have been shown to predict the conformational flexibility of proteins reliably from a single 3D structure [34–36]. The ProFlex code was modified and extended to enable the program to process structures with a large number of atoms and large number of flexible and rigid regions resulting from hydrogen-bond dilution or an extensive network of interactions. The changes accommodated the size of the urease complex and extended the utility of ProFlex for analysis of other very large proteins, including those with multiple subunits. These changes allowed processing of the very large urease structure (~22,000 atoms in the trimer of trimers). The ProFlex software is available to other research groups by request to proflex@sol.bch.msu.edu.

**Materials and methods**

### Protein purification

(UreABC–UreD)₃, (UreABC–UreDF)₃, and urease holoenzyme were produced in E. coli DH5α carrying pKAUD2 [15], E. coli DH5α x pKAUD2/FΔureG [16], or E. coli HMS174(DE3) carrying pKK17 [25] and purified as previously described [30]. HEDG buffer (25 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM DTT, 1% glycerol) was used as a final storage buffer unless noted. The homogeneity of samples was assessed by densitometric analysis (AlphaImager) of Coomassie-stained gels after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) [31]. The expression level of urease subunits in cell extracts was assessed by SDS–PAGE followed by electroblotting the sample onto Immobilon-P polyvinylidene difluoride membrane, probing with anti-K. aerogenes urease antibodies [32], and visualizing with anti-rabbit immunodetec-tion kit. The identity of a band in one sample was examined by Western blot with anti-K. aerogenes UreE antibodies [33].

### Flexibility analysis

We used the graph theoretic algorithm ProFlex to analyze the flexibility of urease (Protein Data Bank (PDB) entry 1FWJ).
aggregation due to radiation damage; none was found. For $(\text{UreABC–UreD})_3$ and $(\text{UreABC–UreDF})_3$, four 4-h runs were summed together, while five 4-h runs were summed together for the native urease complex. These measurements included runs with fresh material and runs in which the sample was exposed for an additional 4 h to check for radiation damage. No artifacts due to radiation damage were observed. Data were reduced, azimuthally averaged and scaled into absolute units (1/cm) according to previously published procedures [39] to provide the 1D intensity profile $I(q)$ vs. $q$, where $q = 4\pi \sin(\theta)/\lambda$, $\theta$ is the scattering angle from the incident beam, and $\lambda$ is the wavelength of the X-ray radiation (1.542 Å).

Small-angle X-ray scattering analysis and modeling

Data were subjected to Guinier analysis [40] for the radius of gyration, $R_g$, and for the pair-distance distribution function $P(r)$. $I(q)$ and $P(r)$ are related through the Fourier transform shown in the following equation

$$P(r) = \frac{1}{2\pi^2} \int_0^\infty q^2 I(q) \cdot \sin(qr) \cdot dr$$

The program GNOM [41] uses an indirect transform method to find $P(r)$ from an input maximum linear dimension, $d_{\text{max}}$. The optimum $d_{\text{max}}$ is found by trial and error to find a solution that best-fits the data and provides an acceptable termination of $P(r)$ at $d_{\text{max}}$. The $P(r)$ fitting also provides a secondary measure of the $R_g$, which is the second moment of $P(r)$.

The program ORNL_SAS [42] was employed to compare the scattering profiles calculated from the urease structure and various models of complexes against the measured SAXS profiles of the enzyme $(\text{UreABC–UreD})_3$ and $(\text{UreABC–UreDF})_3$. To model the $(\text{UreABC–UreD})_3$ and $(\text{UreABC–UreDF})_3$ complexes, ellipsoids were used in place of the unknown structures of UreD and UreF. The structures of the higher-order complexes were built by placing three identical ellipsoids with the $(\text{UreABC})_3$ structure about the same 3-fold symmetry axis around which the trimer of trimers is formed. The translation coordinates were chosen randomly from a range of values that made it possible to produce complexes that extended beyond the experimentally determined $d_{\text{max}}$. To ensure the proper volume for the added proteins, two of the ellipsoidal semiaxes were randomly chosen from a range of 10 to 35 Å, and the proper volume for the added proteins, two of the ellipsoidal extended beyond the experimentally determined volumes based on the amino acid sequence of the subunit. In the event that did not overlap with the $(\text{UreABC})_3$, or the $(\text{UreABC})_3$ plus $(\text{UreABC–UreDF})_3$ structure and the volumes occupied by the ellipsoids were not parameters in the data.

Results

Flexibility analysis of urease

ProFlex [34] was used to analyze the flexibility within the native enzyme trimer of trimers (PDB entry 1PWJ; Fig. 2A and Supplementary Fig. S1A), identifying a total of ~3100 hydrogen bonds and ~1500 hydrophobic interactions. The regions of the protein defined as rigid or flexible were found to vary little with the choice of hydrogen-bond energy cutoff in ProFlex (between $-1$ and $-2$ kcal/mol), defining the set of hydrogen bonds and salt bridges incorporated in the network. In the crystal structure of urease, UreB is anchored by six N-terminal residues that add to the edge of a beta sheet in UreC (Fig. 2B and C, region 1). A salt bridge and at least six hydrophobic interactions between UreB residues 2–8 and UreC residues 6–29 reinforce the attachment (Supplementary Tables S1 and S2). ProFlex predicted UreB residues 11–19 to form a flexible hinge (Fig. 2 Tables S3 and S4) between the N-terminal anchor and the relatively rigid domain formed by UreB residues 20–101. The latter domain includes polar and hydrophobic interactions with UreC (Tables S5 and S6), but these are few in number compared to the interactions with regions 1 and 2 and consistent with the possibility of domain movement. The anchored and hinge residues of the N-terminal region of UreB (residues 1–19) fit into a groove of the N-terminal region of UreC formed by residues C2–C41 (Fig. 2B).

Chemical modification results [28] indicate that UreB Lys76 and UreC Lys382 can be cross-linked when in the $(\text{UreABC–UreDF})_3$ species. This requires bringing their side chains within 10 Å, although they are 50 Å apart in the urease crystal structure. Thus,
we probed whether the flexibility of UreB residues 11–19 would allow these two Lys residues to move to within cross-linking distance while maintaining favorable packing between UreB and UreC. In the first approach, UreB Gly11 and Gly18 were of special interest due to the prevalence of Gly in flexible regions of proteins. This is because Gly residues have no constraints on main-chain bond rotations (Φ and Ψ angle torsions) due to the absence of side chain induced steric hindrance. The torsion angles of UreB Gly11 and Gly18 were manually changed to reduce the distance between UreB Lys76 and UreC Lys382 and attain reasonable packing between UreB and UreC. The resulting distance between the Cα atoms of UreB Lys76 and UreC Lys382 was 19.8 Å, close enough to allow cross-linking of their side chains. This motion involved a rotation of +131° in Φ and +110° in Ψ for Gly11, with 7° changes in both Φ and Ψ for Gly18, creating UreB conformation 1 (Supplementary Fig. S1B). In a second approach, we cut the tether at UreB Gly11, docked UreB Lys76 within cross-linking distance of UreC Lys382 while maintaining good packing between the subunits, and reconnected the tether. This approach created UreB conformation 2 (Supplementary Fig. S1C). A close-up view highlighting the repositioning of UreB to achieve conformation 1 and allow cross-linking is depicted in Fig. 3. Both approaches yielded substantially similar placement of UreB at the periphery of (UreBAC)3 due to the strong constraints placed by maintaining the anchoring interactions of UreB residues 2–10 while meeting the cross-linking distance between UreB Lys76 and UreC Lys382.

Mutagenesis of hinge residues

To directly test the importance of putative UreB hinge region residues Gly11 and Gly18 in urease activation, their codons were independently modified to encode Pro residues that would restrict hinge flexibility. Constructs encoding the G11P and G18P variants of UreB were created and used to substitute for the wild-type sequence in a plasmid containing the complete urease gene cluster. The mutated plasmids were transformed into host E. coli cells, and we monitored their ability to express functional urease activity. The results showed that the G11P and G18P mutations significantly reduced urease activity, indicating that the flexibility of these residues is crucial for the activation of the enzyme.

Fig. 2. Tether and hinge regions between UreB and UreC from the crystallographic structure of urease. (A) The native urease structure, with ribbons colored red for UreA, blue for UreB (except for its hinge and tether to UreC shown in white), and green for UreC. (B) An expanded view of the region encircled in yellow in (A). The N-terminus of UreB (residues 2–8) forms the terminal strand of a beta sheet with UreC. UreB residues 11–19 together with UreC residues 2–6 and 13–41 form a flexible linkage between the main domain of UreB (blue ribbons in (A)) and the disk formed by (UreBAC)3 (red and green ribbons in (A)). Sites relevant to flexibility probing mutations, UreB Pro10, Gly11, and Gly18, are rendered as beads. (C) The same view as (B), colored in terms of ProFlex flexibility analysis of the crystal structure (PDB entry 1FWJ). The N-terminus of UreB partitions from a rigid region (colored blue; region 1) to a flexible hinge (colored gold; region 2) which connects to the globular domain of UreB (shown in blue ribbons in (A)). The terminus of UreC is highly flexible (red), whereas residues in UreC that intervene between regions 1 and 2 are isostatic, or barely rigid, as shown in grey. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Close-up of the repositioning of UreB. The main domain of UreB is proposed to shift from its crystallographic position (dark blue; PDB 1FWJ) to a position (white) in which UreB Lys76 can cross-link with UreC Lys382 (pink CPK spheres), opening access to the active site. The range of motion of UreB hinge residues resulting in this rotation of UreB is shown by the series of blue to lighter blue conformations of residues 11–19 between the UreB crystallographic and cross-linked open positions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
and urease overexpression was shown to be comparable in the control and mutant strains by using Western blots (data not shown). Urease activity in cell extracts containing the G18P variant of UreB was similar to that for extracts containing wild-type enzyme, indicating the flexibility of residue 18 is not critical to urease activation. In contrast, extracts containing the G11P mutant displayed 15–50% (depending on the preparation) of the activity of the control strain. This result suggests that protein dynamics requiring the flexibility of residue 11 are important to the metal-center assembly process.

Urease-containing UreB G11P was purified from the mutant strain and subjected to metal analysis. Whereas control enzyme exhibits a specific activity of \( 2200 \pm 200 \text{ mol min}^{-1} \text{ (mg protein)}^{-1} \) and contains 2.1 ± 0.3 nickel ions per active site [44], the purified UreB G11P variant protein possessed a specific activity of approximately 440 \text{ mol min}^{-1} \text{ (mg protein)}^{-1} \) and only contained 1.67 nickel ions per active site (single determination with an estimated error of <10%). For comparison, previous experiments showed that when (UreABC)\(_3\) was incubated with nickel plus bicarbonate \textit{in vitro}, 2.13 to 1.74 nickel ions were present per active site with the activated enzyme yielding specific activities of 0 and 442 \text{ mol min}^{-1} \text{ (mg protein)}^{-1} \) [20], as opposed to 2200 \text{ mol min}^{-1} \text{ (mg protein)}^{-1} \); thus, high nickel content can be associated with inactive protein. These results suggest both a deficiency in nickel incorporation and formation of a less effective dinuclear site in the mutant protein. Significantly, the mutant urease protein was resolved into two fractions during phenyl-Sepharose chromatography (Fig. 4). The highly purified urease analyzed above was obtained by elution with buffer lacking salt, as in the case of wild-type enzyme. In addition, a nearly inactive urease-containing fraction was obtained by subsequent washing of the resin with water. The second pool of urease contained four major contaminating proteins that co-migrated with UreD (Mr 29,807), UreG (Mr 21,943), UreF (Mr 25,221), and UreE (Mr 17,558) (note that the peptides do not migrate precisely according to their known size). A Western blot analysis with anti-UreE antibodies (data not shown) confirmed the identity of UreE in this sample. The finding of this newly identified complex is compatible with the need for flexibility in the hinge region of UreB to achieve accessory protein dissociation. The deleterious effects on urease activity, nickel content, and accessory protein dissociation that come from restricting the motion of UreB Gly11 by Pro substitution are consistent with the observation that large changes in main-chain \( \Phi \) and \( \Psi \) values of UreB Gly11 are needed to place Lys76 of this subunit within cross-linking distance of Lys382 in UreC. The neighboring residue, UreB Pro10, already limits the accessible \( \Phi \) angles so the G11P mutant would severely restrict the conformations available to the hinge. These results are consistent with a hinge-like motion of UreB relative to UreC upon binding of UreD and UreF, allowing access to the active site for activation.

Small-angle X-ray scattering measurements and analyses

SAXS data were collected for native urease, (UreABC–UreD)\(_3\), and (UreABC–UreDF)\(_3\) (Fig. 5). Instrument stability issues, primarily due to temperature fluctuations in the facility, caused the differences in usable minimum \( q \) shown in the graph. The inset curves in Fig. 5 are the Guinier regions for the three data sets, and correspond to \( R_g \) of 32.7 ± 2.4, 40.3 ± 2.3, and 50.6 ± 2.5 Å for the respective species. In all cases, the Guinier regions are linear, indicative of monodisperse scattering particles. The data do not display artifacts due to interparticle interference, which manifests as a significant downturn at low \( q \)-values. The \( P(r) \) curves derived from the SAXS data (Fig. 6) indicate increasing size with increasing number of components. The \( R_g \) for urease determined from the \( P(r) \) fitting was 35.7 ± 0.8 Å, with a \( d_{\text{max}} \) of 95 ± 5 Å. The values of \( R_g \) for the (UreABC–UreD)\(_3\) and (UreABC–UreDF)\(_3\) complexes were 44.9 ± 0.7 and 53.7 ± 1.4 Å, respectively. The \( d_{\text{max}} \) of the (UreABC–UreD)\(_3\) complex was 130 ± 8 Å, while that of the (UreABC–UreDF)\(_3\) complex was 155 ± 10 Å. The agreement between the Guinier- and GNOM-derived \( R_g \) values is reasonable considering the very different methods of obtaining the values and estimating the uncertainties.

**Fig. 4.** Two pools of the UreB G11P mutant urease resolved by phenyl-Sepharose chromatography. Molecular weight standards (Std), the purified active mutant urease (lane 1), and the very low activity complex containing mutant urease (lane 2) were examined by SDS–PAGE using a 13.5% acrylamide gel and stained with Coomassie brilliant blue.

**Fig. 5.** \( I(q) \) curves derived from the scattering data for urease (■), (UreABC–UreD)\(_3\), (○), and (UreABC–UreDF)\(_3\) (▲). The lines are the model fits to the data using the crystal structure of urease (PDB 1FWJ) (solid line), with UreB Gly11/Gly18 torsionally adjusted to allow cross-linking of UreB Lys76 to UreC Lys382 (dashed line), and UreB docked to UreAC from the crystal structure, allowing cross-linking of UreB Lys76 to UreC Lys382 (dotted line). The curves have been offset by a multiplicative factor for clarity. The inset plot shows the Guinier regions and fit lines for the three measured profiles. Again, the curves have been offset for clarity, and the region of data covered by the line indicates the range of data used for the fitting.
Models of the activation complexes

The intensity profile calculated from the wild-type urease crystal structure [3] using the program ORNL_SAS [42] is shown with the data in Fig. 5. The agreement between the measured data and the simulated profile is excellent, having a $\chi^2$ of 0.493. The fit of the model intensity profile to the data across the entire q-range is excellent.

Models of (UreABC–UreD)$_3$ were generated by adding UreD ellipsoids to the wild-type urease structure and to (UreABC)$_3$ with the two alternative UreB conformations: torsionally adjusted and docked. Ellipsoids were used because no structure or homology model is available for any UreD. In all cases, the overall structures of the final complexes were very similar. The best models had UreD ellipsoids added to the vertices of (UreABC)$_3$ near the UreB subunit such that the total structure has a planar, triangular character (Supplementary Fig. S2). The best three model intensity profiles for the three different starting structures have $\chi^2$ values of 0.218, 0.252, and 0.224 when starting with the native structure, torsionally adjusted UreB, and docked UreB, respectively. In all cases, the fits of the profiles to the data are excellent in light of the experimental uncertainties shown in Fig. 5 and suggest that all of the structures are reasonable. It is important to note that the three models all have the same general shape, which is the most reliable result of the modeling considering the method of building the models and the lack of a high-resolution structure of UreD. The addition of UreD results in a planar, triangular structure. The (UreABC–UreD)$_3$ results are in agreement with UreB interacting with UreB as suggested by chemical cross-linking [28].

Models of (UreABC–UreDF)$_3$ were created by adding ellipsoids to represent appropriate molecular volumes of UreD and UreF to the (UreABC)$_3$ crystal structure and the torsionally adjusted and docked models produced by the flexibility modeling. As above, no high-resolution structure or model is available for UreF; however, a homology model was reported for UreF from Bacillus pasteurii [45]. The 202 residue B. pasteurii protein is 32% identical over only 91 residues of the 224 amino acid K. aerogenes UreF. For this reason ellipsoids were chosen to represent this protein in the modeling, as well. The models produced using the two alternative UreB conformations (torsionally adjusted and docked) are similar, and in fact resulted in similar placements of UreD and UreF in the best-fitting SAXS models (shown for the docked conformation in Fig. 7). The best UreB conformation 1 (torsionally adjusted) and UreB conformation 2 (docked) structures fit the scattering data very well, as well as account for the cross-linking results, and have $\chi^2$ of 0.093 and 0.094, respectively, as compared to the $\chi^2$ of 0.096 observed for the model produced from the native UreB structure. The fit of the model profiles to the data are all excellent, as can be seen in Fig. 5, so it is not possible to discriminate between the SAXS models for the reasons provided above. The overall shape of the complex, which can be reliably extracted from the data, is very consistent between the three models (Supplementary Fig. S3), having a planar, triangular character with the additional mass corresponding to UreD and UreF located near the vertices, almost coplanar with the rest of the structure. The UreF ellipsoids are near the UreD ellipsoids in all of the models, rather than being spatially separated. The model depicted in Fig. 7 appears to build on the models of (UreABC–UreD)$_3$, with the UreD and UreF ellipsoids positioned pairwise at the vertices of the (UreABC)$_3$ structure. In this case UreB, UreD, and UreF essentially add onto the edge of the disk formed primarily by the UreC trimer, in which UreA forms the hub (Fig. 2A). These structures are consistent with immunological results that show anti-UreD antibodies recognize UreD within (UreABC–UreD)$_3$, but not within (UreABC–UreDF)$_3$, suggesting that UreF partially masks UreD [16]. In addition, these results are consistent with cross-linking between UreF and UreB [28].

Discussion

In this work we combined multi-scale modeling and sparse experimental constraints to obtain insight into a flexible molecular assembly, the urease activation complex. In particular, we used flexibility analysis to provide evidence that the major domain of UreB can move in a hinge-like motion to allow sufficiently close juxtaposition of UreB Lys76 with UreC Lys382 to form a chemical cross-link between these residues, as previously reported [28]. The UreB G11P variant, which is likely to rigidify the hinge region, was shown to lead to reduced levels of urease activation and lower

Fig. 6. Profiles of the scattering data for urease (■), (UreABC–UreD)$_3$ (○), and (UreABC–UreDF)$_3$ (▲). To simplify comparison, the curves have been scaled to have a value of 1.0 at the peak.

Fig. 7. Predicted positioning of UreD and UreF relative to the crystallographic structure of (UreABC)$_3$, based on best-fit models to SAXS data. The best-fit models resulted in packing of UreD and UreF against UreB near a vertex of the (UreABC)$_3$ disk. A representative example is illustrated. Urea, UreB, and UreC are rendered in red, yellow, and green ribbons, respectively. UreB and UreF from SAXS results are rendered as solid ellipsoids colored purple and magenta, respectively. The non-interpenetrating volumes of the UreD and UreF ellipsoids accounts for the appropriate molecular weight of each subunit. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
nickel content while also sequestering a significant portion of the urease apoprotein in an ineffective activation complex that includes all four of the known K. aerogenes accessory proteins. The larger impact observed for the G11P variant compared to the H. pylori UreA subunit (corresponding to a fusion of UreA and UreB in the K. aerogenes enzyme) contains a fold that matches the K. aerogenes UreB fold, but also contains residues that add to one side of this shared fold in a similar position to where we predict UreD and UreF bind. A viral protein (PDB entry 1C5E) also contains this shared fold, with an additional domain in the same region as the added domain in H. pylori UreA. Thus, both other proteins that share the UreB domain fold with K. aerogenes urease use this domain as a molecular interface, suggesting that this region of UreB has evolved to interact with other domains or proteins. This supports its role in K. aerogenes UreB as a docking interface for UreD or UreF.

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Appendix A. Supplementary data


References


