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Evidence for the Presence of Urease Apoprotein Complexes Containing UreD, UreF, and UreG in Cells That Are Competent for In Vivo Enzyme Activation

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In vivo activation of *Klebsiella aerogenes* urease, a nickel-containing enzyme, requires the presence of functional UreD, UreF, and UreG accessory proteins and is further facilitated by UreE. These accessory proteins are proposed to be involved in metallocenter assembly (M. H. Lee, S. B. Mulrooney, M. J. Renner, Y. Markowicz, and R. P. Hausinger, J. Bacteriol. 174:4324–4330, 1992). A series of three UreD-urease apoprotein complexes are present in cells that express *ureD* at high levels, and these complexes are thought to be essential for in vivo activation of the enzyme (I.-S. Park, M. B. Carr, and R. P. Hausinger, Proc. Natl. Acad. Sci. USA 91:3233–3237, 1994). In this study, we describe the effect of accessory gene deletions on urease complex formation. The *ureE*, *ureF*, and *ureG* gene products were found not to be required for formation of the UreD-urease complexes; however, the complexes from the *ureF* deletion mutant exhibited delayed elution during size exclusion chromatography. Because these last complexes were of typical UreD-urease sizes according to native gel electrophoretic analysis, we propose that UreF alters the conformation of the UreD-urease complexes. The same studies revealed the presence of an additional series of urease apoprotein complexes present only in cells containing *ureD*, *ureF*, and *ureG*, along with the urease subunit genes. These new complexes were shown to contain urease, UreD, UreF, and UreG. We propose that the UreD-UreF-UreG-urease apoprotein complexes represent the activation-competent form of urease apoprotein in the cell.

Urease is a nickel-containing enzyme that catalyzes the hydrolysis of urea to form ammonia and carbamate (3, 18). The latter compound spontaneously decomposes to generate a second molecule of ammonia and carbon dioxide. Many recent studies have focused on characterization of bacterial urease, a virulence factor that is implicated in the formation of urinary stones, gastric ulceration, and other medical complications (for a review, see reference 11). In addition to providing more information about the enzyme itself, biochemical and molecular biological studies of bacterial urease have revealed the presence of a surprisingly complex metallocenter assembly process that is required for enzyme activation. The key features of this maturation pathway are summarized below.

Klebsiella aerogenes urease apoprotein is composed of three distinct subunits (α, β, and γ, encoded by the *ureC*, *ureB*, and *ureA* genes, respectively [12]) arranged in an $(\alpha\beta\gamma)_3$ structure. The protein can be partially activated in vitro by providing carbon dioxide in addition to nickel ions (15). The apoprotein is hypothesized to react with carbon dioxide to form a metallocenter ligand. This proposal is based on urease activation kinetics in the presence of varying levels of carbon dioxide and nickel ion and on the precedent of ribulose bisphosphate carboxylase/oxygenase. The latter protein is known to generate a lysine carbamate that acts as a ligand for binding the essential magnesium ion of that enzyme (for a review, see reference 2). In contrast to this simple in vitro system for urease apoprotein activation, the in vivo process for urease maturation requires participation by the products of at least four accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*). Deletion mutants missing portions of *ureD*, *ureF*, or *ureG* synthesize only urease apoprotein, and *ureE* deletion mutants synthesize urease with depressed activities and correspondingly reduced nickel contents (6). Overexpression of *ureD* in the presence of the other urease genes and the absence of added nickel ions leads to the formation of a series of UreD-urease apoprotein complexes (14). These complexes correspond to the native $(\alpha\beta\gamma)_3$ urease molecule associating with one, two, or three molecules of UreD. These species are activated in vitro by the addition of nickel ions in a carbon dioxide-dependent manner (14, 15). Activation is accompanied by dissociation of UreD from the holoprotein. The role for UreD and the significance of the UreD-urease complexes remain unclear. The *ureE* gene product has been purified and partially characterized (7). The dimeric UreE protein binds approximately six nickel ions with a dissociation constant of ~10 μM. This interaction is highly selective for nickel ions, and the holoprotein form of UreE has been suggested to serve as a nickel donor during urease apoprotein activation. Although essential for in vivo maturation of urease, the roles of UreF and UreG are not known. The latter protein, however, is related in sequence to HypB, a GTPase that functions in nickel processing for activation of hydrogenase (9, 16).

In this study, we set out to test the hypothesis that UreF or UreG may function in the assembly of the UreD-urease complexes. By examination of deletion mutants, this hypothesis was proven to be incorrect. During the course of these studies, however, the presence of UreF was found to affect the conformation of the UreD-urease complexes, resulting in a change in their chromatographic behavior. Furthermore, evidence for the presence of a series of urease apoprotein complexes that include UreD, UreF, and UreG was obtained. Because all of these components are required for in vivo activation of urease, we propose that these complexes are the key species involved in enzyme maturation in vivo.

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MATERIALS AND METHODS

Cell growth and disruption. *Escherichia coli* DH5 cells carrying pKAU17 (13) (containing the intact wild-type *K. aerogenes* urease gene cluster), pKAUD2 (14) (containing the intact gene cluster with enhanced expression of *ureD*), pKAUΔD-1, pKAUΔE-1, pKAUΔF, and pKAUΔG-1 (deletion derivatives of pKAU17 lacking portions of *ureD*, *ureE*, *ureF*, and *ureG*, respectively) (6), or pKAUDABC (pKAU17 lacking *ureE*, *ureF*, and *ureG* constructed by deleting a fragment from the *Ksp*I site of *ureE* to the *Rsr*II site of *ureG*) were grown in Luria-Bertani (LB) medium containing no added nickel to stationary phase. Under these conditions, urease activity was nearly undetectable and the apoprotein was synthesized. The harvested cultures were resuspended in PED buffer (20 mM phosphate [pH 7.0], 0.1 mM EDTA, 0.1 mM dithiothreitol) containing 1 mM phenylmethylsulfonyl fluoride and disrupted by three passages through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 18,000 lb/in^2. Cell extracts were obtained after centrifugation (100,000 × *g* for 60 min) at 4°C. To stabilize the extracts during storage, glycerol was added to a concentration of 10%.

Urease activity assays. Urease activities were assayed in 25 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES; pH 7.75) buffer containing 50 mM urea and 0.5 mM EDTA. Linear regression analysis of the released ammonia, determined by conversion to indophenol (17), versus time yielded initial rates. One unit of activity is defined as the amount of enzyme required to degrade 1 μmol of urea per min at 37°C. Protein concentrations were assessed by the spectrophotometric assay of Lowry et al. (8), with bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out by using the buffers of Laemmli (4). Nondenaturing gels utilized the same buffers without SDS. Gels were stained with Coomassie brilliant blue or, in the case of selected native gels, blotted onto nitrocellulose membranes, probed with anti-*K. aerogenes* urease (13) or anti-UreG antibodies (5), and developed by using anti-rabbit immunoglobulin G–alkaline phosphatase conjugates (1). Kinetic analysis of the activation process by electrophoresis involved the incubation of samples in activation buffer (1% glycerol, 8 μM dithiothreitol, 8 μM EDTA, 0.8 mM phosphate, 25 mM HEPES [pH 7.9]) and addition of 5 mM EDTA at selected time points to quench activation by sequestering nickel ions, followed by native gel electrophoresis. Enzyme activities of cut gel fragments were measured after a 16-h incubation in the enzyme assay buffer at 4°C.

Analysis of UreD-urease apoprotein complexes by size exclusion chromatography. Cell extracts prepared from *E. coli* DH5(pKAU17), DH5(pKAU17ΔE-1), DH5(pKAU17ΔF), and DH5(pKAU17ΔG-1) were subjected to chromatography on a Sepharose 6 column (2.6 by 50 cm) equilibrated with PED buffer. The 4-ml fractions were examined for the presence of urease and urease complexes by Western blotting (immunoblotting) as described above.

Partial purification of the B complexes. Cell extracts from *E. coli* DH5 carrying pKAU17 or pKAUD2 were subjected to chromatography on a column of DEAE-Sepharose at 4°C in PEDG buffer (18 mM phosphate [pH 7.0], 0.09 mM EDTA, 10% glycerol, 0.09 mM dithiothreitol). The proteins were eluted in the same buffer with a linear salt gradient to a concentration of 1.0 M KCl. The fractions containing the B protein complexes (see Results and Discussion), as determined by using native polyacrylamide gel electrophoresis, were pooled, dialyzed against PEDG buffer, and applied to a Mono-Q HR 10/10 column equilibrated in the same buffer. The proteins were eluted with a linear salt gradient to a 1 M KCl concentration in PEDG buffer with the B complexes eluting at a concentration of ≈0.5 M KCl.

N-terminal sequencing of UreF. The partially purified B complexes from cell extracts of *E. coli* DH5 carrying pKAU17 were subjected to SDS electrophoresis on a 10% polyacrylamide gel and transferred to a sheet of Pro-Blot membrane (Applied Biosystems, Foster City, Calif.) by standard procedures (10). The band corresponding in mobility to that expected for UreF was cut from the membrane and analyzed by using an Applied Biosystems 477A automated sequencer in the Michigan State University Macromolecular Structure Facility.

RESULTS AND DISCUSSION

Effects of UreE, UreF, and UreG accessory proteins on formation of UreD-urease apoprotein complexes. *E. coli* cells containing the *K. aerogenes* urease genes and grown in LB medium lacking added nickel ions are known to produce urease apoprotein that is present in a series of complexes containing 0, 1, 2, or 3 molecules of UreD, depending on the *ureD* expression level (14). For reference, these protein complexes are indicated in Fig. 1 for cell extracts containing the wild-type gene cluster (lanes 1 and 4) and a *ureD* overexpression mutant (lane 3). To test if another accessory gene (*ureE*, *ureF*, or *ureG*) is required for formation of the UreD-urease apoprotein complexes, plasmid pKAUDABC containing only *ureD*, *ureA*, *ureB*, and *ureC* was constructed (see Materials and Methods). *E. coli* cells conveying this plasmid or previously described plasmids carrying smaller deletions within *ureE*, *ureF*, or *ureG* (6) were shown to possess UreD-urease species (Fig. 1, lanes 2 and 6 to 8). In contrast, a *ureD* deletion mutant failed to produce these complexes (Fig. 1, lane 5). These results indicated that formation of the UreD-urease apoprotein complexes does not require other accessory genes.

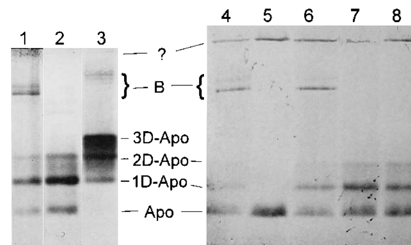

FIG. 1. Western blot comparison of urease-containing species in selected cell extracts subjected to native polyacrylamide gel electrophoresis. Extracts of *E. coli* DH5(pKAU17) containing the wild-type *K. aerogenes* gene cluster (lanes 1 and 4), *E. coli* DH5(pKAUDABC) containing only *ureD*, *ureA*, *ureB*, and *ureC* genes (lane 2), *E. coli* DH5(pKAUD2) containing the intact gene cluster with enhanced *ureD* expression (lane 3), *E. coli* DH5(pKAU22ΔD-1) lacking a functional *ureD* gene (lane 5), *E. coli* DH5(pKAU17ΔE-1) with a deletion in *ureE* (lane 6), *E. coli* DH5(pKAU17ΔF) with a deletion in *ureF* (lane 7), and *E. coli* DH5(pKAU 17ΔG-1) with a deletion in *ureG* (lane 8) were analyzed on a 6% (lanes 1 to 3) or 5% (lanes 4 to 8) native gel, electroblotted onto a nitrocellulose membrane, treated with antiurease antibodies, and developed by using an anti-immunoglobulin G–alkaline phosphatase conjugate. Apo denotes the migration position of the urease apoprotein, whereas XD-Apo (with X representing 1, 2, or 3) denotes the position of the three UreD-urease apoprotein complexes. A second or B series of complexes (B) and additional cross-reactive material (?) are indicated.

While comparing the properties of the UreD-urease complexes in samples from cells containing the intact gene cluster and various deletion mutants, we observed a difference in the *ureF* deletion mutant that provides evidence for a role of UreF in complex formation. The major UreD-urease apoprotein complex in cell extracts prepared from *E. coli* DH5(pKAU17) (other UreD-urease complexes were not detected in this experiment) eluted earlier from a Sepharose 6 column than did the apoprotein, consistent with a larger hydrodynamic radius for UreD-urease than for urease alone (data not shown). Similar elution patterns were observed for extracts from cells carrying pKAUΔE-1 (Fig. 2A) or pKAUΔG-1 (data not shown)

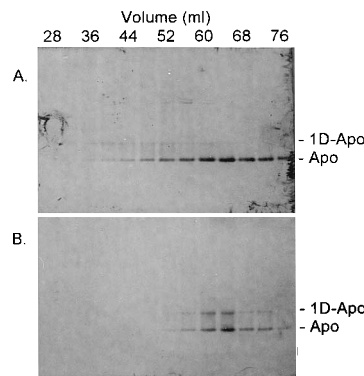

FIG. 2. Western blot comparison of fractions from Sepharose 6 column chromatography of selected cell extracts. Cell extracts were prepared from *E. coli* DH5(pKAU17ΔE-1) with a deletion in *ureE* (A) and *E. coli* DH5(pKAU17ΔF) with a deletion in *ureF* (B) and applied to a Sepharose 6 column. Fractions were analyzed on a 6% native gel, and urease-containing species were visualized by immunological methods. The apoprotein migration positions are indicated on the right.

(i.e., *ureE* and *ureG* deletion mutants). Surprisingly, the major UreD-urease apoprotein complex in extracts from cells carrying pKAUΔF, a *ureF* deletion mutant, eluted at the same position as the urease apoprotein (Fig. 2B). Because the UreD-urease complex in this construct coelectrophoreses with the major UreD-urease complexes in the other constructs, it is unlikely to have a significantly altered hydrodynamic radius. Rather, we suggest that the UreF protein alters the conformation of the UreD-urease apoprotein complex to reduce its hydrophobicity or otherwise decrease nonspecific interactions with the Sepharose resin. It is possible that such a conformational change is related to the decreased level of in vitro urease activation observed for *E. coli* DH5(pKAUΔF) cell extracts compared with levels for a control sample or samples possessing *ureE* or *ureG* deletions (14).

Evidence for the presence of a series of urease apoprotein complexes containing UreD, UreF, and UreG. In addition to the three known UreD-urease apoprotein complexes reported earlier (14) and described above, several faint bands of much slower mobility that contain antiurease antibody cross-reactive material were detected for cell extracts from *E. coli* DH5 conveying pKAU17, pKAU17ΔE-1, and pKAUD2 in Western blotting studies (lanes 1, 3, 4, and 6 of Fig. 1). The bands were designated B in the figures in this report because they constituted a second or B series of complexes that were distinct from the faster-migrating UreD-urease complexes already discussed. These bands are unlikely to arise from artifactual cross-linking of abundant proteins via disulfide bands, as shown by their continued presence in buffers containing dithiothreitol. Of interest, four species of B complex, a number similar to that of the UreD-urease apoprotein complexes, appeared to be present. Furthermore, the more slowly migrating bands of the B complexes were more intense for the cells that overexpress *ureD* [*E. coli* DH5(pKAUD2)], whereas the more rapidly migrating species were more pronounced in the extracts from *E. coli* DH5(pKAU17) cells. Again, this pattern followed that observed for the UreD-urease complexes. (An even more slowly migrating band or series of bands that appeared to contain urease also was seen for cell extracts from these *E. coli* cells [indicated by the question mark in Fig. 1] but will not be further discussed.) Treatment of the extracts by dialysis or with ATP, GTP, or high salt (up to 1 M KCl) concentrations failed to dissociate the B complexes into their faster migrating components (data not shown). Importantly, B bands were not detected in the *ureEFG* deletion mutant (Fig. 1, lane 2) or in the individual *ureD*, *ureF*, and *ureG* deletion mutants (Fig. 1, lanes 5, 7, and 8). It is very intriguing that cells found to be activation competent in vivo possessed the B complexes, whereas *ureD*, *ureF*, and *ureG* deletion mutants, which are not competent for in vivo activation (6), did not.

The B complexes were enriched from extracts of cells containing pKAU17 and pKAUD2 by using DEAE-Sepharose and Mono-Q column chromatographies. The B species did not separate from the UreD-urease apoprotein complexes when Sepharose 6 size exclusion column chromatography was used. This result indicates that the B complexes are not significantly larger than the UreD-urease complexes and that size differences do not account for the observed differences in electrophoretic behavior of these two series of complexes during native polyacrylamide gel electrophoresis. Although the peaks overlapped, the peak fractions of the UreD-urease complexes eluted before the peak fractions of the B complexes during ion exchange chromatography, as shown for the pKAUD2-derived sample (Fig. 3A). When the same fractions were examined by denaturing gel electrophoresis (Fig. 3B), it was clear that the fractions containing the B complexes possessed several bands

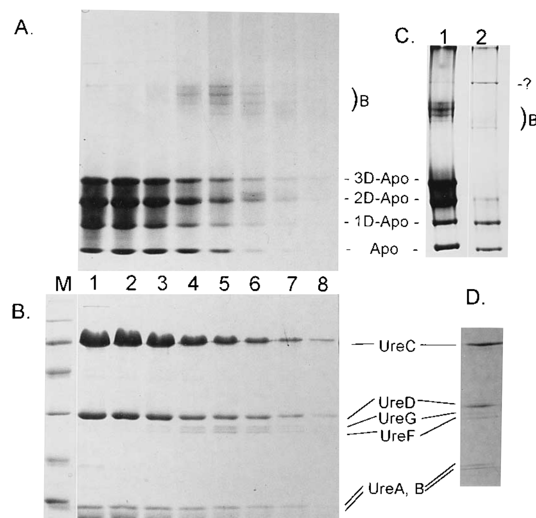

FIG. 3. Partial purification and characterization of the urease-containing B complexes. (A) Native 5% polyacrylamide gel electrophoretic analysis of Mono-Q fractions eluting at between 0.43 and 0.51 M concentrations of KCl from *E. coli* DH5(pKAUD2) cells possessing an intact urease gene cluster and elevated levels of UreD. (B) SDS-polyacrylamide gel (13.5% polyacrylamide) electrophoretic analysis of the Mono-Q fractions used for panel A. Lane M, molecular weight markers (phosphorylase *b*, M_r of 92,500; bovine serum albumin, M_r of 66,200; ovalbumin, M_r of 45,000; carbonic anhydrase, M_r of 31,000; soybean trypsin inhibitor, M_r of 21,500; and lysozyme, M_r of 14,400). (C) Nondenaturing polyacrylamide gel electrophoretic analyses of Mono-Q pools of B complex-containing fractions from *E. coli* DH5(pKAUD2) (lane 1) or *E. coli* DH5(pKAU17) (lane 2). (D) Two-dimensional gel electrophoretic analysis of the B complex. The native gel portion containing the B complexes of the Mono-Q pool of *E. coli* DH5(pKAUD2) was excised, denatured, and subjected to SDS-polyacrylamide gel electrophoresis. All gels were visualized by Coomassie blue staining. Apoprotein migration positions are indicated as described in the Fig. 1 legend. The lanes contain fractions from a Mono-Q elution.

in addition to those corresponding to urease and UreD. The pooled fractions from cells containing pKAU17 also exhibited bands corresponding to the B complexes (Fig. 3C, lane 2), and these fractions also appeared to possess the additional peptides (data not shown). Because the fractions contained both the UreD-urease complexes and the B complexes, the stoichiometry of these novel peptides relative to UreD or the urease subunits could not be assessed. One of these additional bands for samples derived from cells containing pKAU17 or pKAUD2 was demonstrated to be UreG by a Western blotting experiment using anti-UreG antibodies (data not shown). The band immediately below UreG on the SDS-polyacrylamide gel in the sample from cells containing pKAU17 was excised and shown to have an N-terminal amino acid sequence (a mixture of Met-Ser-Thr-Ala-Glu-Gln and the same sequence without the Met) that agreed with that deduced for UreF from the DNA sequence (12). Bands corresponding to the B complexes from partially purified samples derived from *E. coli* DH5(pKAUD2) (e.g., as shown in Fig. 3C, lane 1) were excised from a native gel and reelectrophoresed on a denaturing gel. Bands migrating at positions appropriate to both UreG and UreF, as well as the urease subunits and UreD, were shown to be present (Fig. 3D). Thus, we suggest that the B complexes are composed of urease apoprotein, UreD, UreF, and UreG.

Urease apoprotein associated with the UreD-UreF-UreG-urease complexes was shown to be capable of being activated. The UreD-UreF-UreG-urease apoprotein complexes present in the Mono-Q pool from *E. coli* DH5(pKAUD2) were found to undergo a change in electrophoretic mobility when the sam-

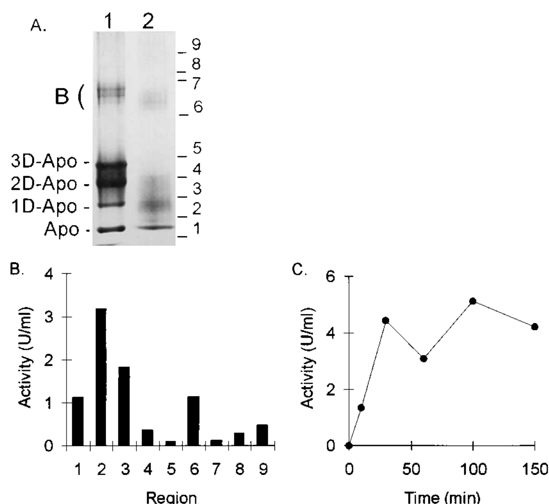

FIG. 4. Activation of urease apoprotein associated with the B complexes. (A) The Mono-Q pool containing the B complexes (parenthesis) of *E. coli* DH5(pKAUD2) (lane 1) was incubated in activation buffer for 100 min (lane 2) at 37°C in the presence of 0.1 mM $NiCl_2$, and aliquots of each sample (31 and 15.5 μg, respectively) were analyzed by native gel electrophoresis. Regions are numbered on the right, and apoprotein migration positions are indicated on the left as described in the Fig. 1 legend. (B) The samples described for panel A were incubated for 30 min prior to electrophoresis, the gel was sliced into the indicated nine sections, and the urease activity in each fragment was measured. (C) The level of activity was measured in gel slices corresponding to region 6 for samples that were incubated in activation buffer for the indicated times.

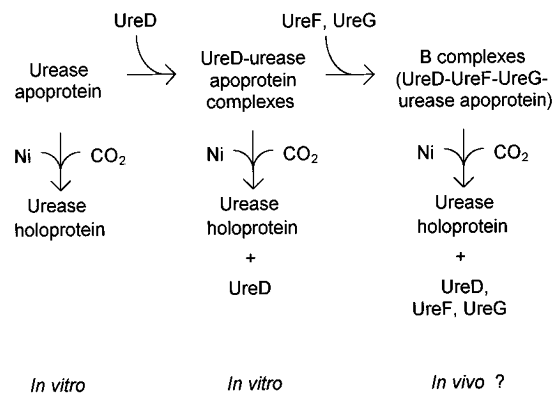

FIG. 5. Speculative model comparing the in vitro and in vivo activation processes for urease activation. Although urease apoprotein or UreD-urease apoprotein complexes can be activated in vitro (14, 15), the presence of functional *ureF* and *ureG* genes is required for in vivo activation. Evidence presented in this paper indicates the existence of a series of UreD-UreF-UreG-urease apoprotein (B) complexes. We propose that these B complexes correspond to the enzyme species that are competent for in vivo activation.

ple was incubated with 100 μM $NiCl_2$ (Fig. 4A). The multiple UreD-UreF-UreG-urease apoprotein complex bands appeared to collapse to form faster-migrating B complexes. This behavior is reminiscent of that reported for activation of the UreD-urease apoprotein complexes (14). No change in electrophoretic behavior was observed when the sample was incubated in the absence of added nickel ions. Individual gel slices derived from a partially activated sample were measured for enzyme activity. The UreD-UreF-UreG-urease complex region was found to possess significant levels of activity that were comparable to those of the regions containing much more protein that was associated with the urease holoprotein and the UreD-urease complexes (Fig. 4B). The rate of enzyme activation in the appropriate region of the gel containing the UreD-UreF-UreG-urease apoprotein complexes was assessed by measuring activities for samples that were incubated for different lengths of time, and the process appeared to be complete in ~30 min (Fig. 4C). Importantly, activation of the UreD-UreF-UreG-urease apoprotein complexes in the gel was found to be more rapid than that of the mixture of apoenzyme forms found in the Mono-Q fractions. The K_m value (± the standard error) determined for the enzyme activity in the gel slice from region 6 was 10.1 ± 0.8 mM. This value is a little higher than that of a typical urease holoprotein (3.0 ± 0.8 mM, measured in the same way, or 2.3 ± 0.2 mM, measured for urease in solution). It is possible that the other proteins that are present in the activated complex slightly hinder access to the active site.

Conclusions. Urease apoprotein alone can be activated in vitro by providing nickel in the presence of carbon dioxide (15). In addition, UreD-urease apoprotein complexes are also activation competent in vitro in a carbon dioxide-dependent manner (14, 15). However, neither the apoprotein alone nor the presence of UreD and urease subunits is sufficient for forming active enzyme in the cell (6). Here, we have shown that the UreD-urease complexes can form in the absence of functional UreE, UreF, and UreG. Of possible relevance to the role for UreF, we have found that the presence of this protein affected the conformation of the UreD-urease complexes. Moreover, we have provided evidence for the presence of complexes that are composed of UreD, UreF, UreG, and urease apoprotein. It is intriguing that UreD-UreF-UreG-urease apoprotein complexes have been found only in the activation-competent cells that contain the wild-type plasmid, the *ureD* overexpressor, or the *ureE* deletion mutant. On the basis of these results, we propose that UreD-UreF-UreG-urease apoprotein complexes are required for in vivo activation of urease, as illustrated in Fig. 5. In this model, nickel may be provided as a complex with UreE, previously shown to be a nickel-binding protein (7). The roles for UreD, UreF, and UreG remain elusive; however, it is reasonable to suggest that they are involved in delivery of carbon dioxide or nickel ions (e.g., by facilitating interactions of the complex with UreE). It is hoped that this speculative model for urease activation will stimulate further studies directed at unraveling the mechanism of urease metallocenter assembly.

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