Following movement of domain IV of elongation factor G during ribosomal translocation

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Translocation of mRNA and tRNAs through the ribosome is catalyzed by a universally conserved elongation factor (EF-G in prokaryotes and EF-2 in eukaryotes). Previous studies have suggested that ribosome-bound EF-G undergoes significant structural rearrangements. Here, we follow the movement of domain IV of EF-G, which is critical for the catalysis of translocation, relative to protein S12 of the small ribosomal subunit using single-molecule FRET. We show that ribosome-bound EF-G adopts distinct conformations corresponding to the pre- and posttranslocation states of the ribosome. Our results suggest that, upon ribosomal translocation, domain IV of EF-G moves toward the A site of the small ribosomal subunit and facilitates the movement of peptidyl-tRNA from the A to the P site. We found no evidence of direct coupling between the observed movement of domain IV of EF-G and GTP hydrolysis. In addition, our results suggest that the pretranslocation conformation of the EF-G–ribosome complex is significantly less stable than the posttranslocation conformation. Hence, the structural rearrangement of EF-G makes a considerable energetic contribution to promoting tRNA translocation.

Significance

The ribosome synthesizes proteins in all living organisms. During the process of protein synthesis, tRNAs and mRNA move through the ribosome, and this movement is catalyzed by the binding of a protein called elongation factor G (EF-G). The mechanism of EF-G–induced translocation of mRNA and tRNAs is not fully understood. In this work, we show that EF-G undergoes structural rearrangements and adopts at least two distinct conformations during translocation. Our work provides new insights into how EF-G promotes tRNA and mRNA translocation.

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translocation, revealed EF-G bound to the ribosome in a conformation similar to previously observed conformations (8). However, domain IV of EF-G was not fully docked into the A site of the small subunit because of a large (−18°) swiveling of the 30S head. In the other cryo-EM structure of the EF-G–ribosome complexes, the ribosome was trapped in the pretranslocation state using an inhibitor of translocation, the antibiotic viomycin (9). Here, EF-G was observed in a previously unobserved conformation: in this structure, the tip of domain IV of EF-G is located ~20 Å away from the A site compared with the posttranslocation conformation. Although this cryo-EM structure provides compelling evidence of EF-G rearrangement during translocation, the movement of domain IV of EF-G relative to the 30S A site has yet to be observed in solution. Thermodynamic and kinetic descriptions of EF-G dynamics during translocation are also lacking. Furthermore, other conformations of ribosome-bound EF-G may exist but have escaped detection by cryo-EM and X-ray crystallography. Therefore, a complete understanding of EF-G dynamics during translocation remains elusive.

Here, we probe the structural rearrangements of ribosome-bound EF-G in solution by following the movement of domain IV of EF-G relative to the A site of the small ribosomal subunit using smFRET and total internal reflection microscopy. We show that domain IV of ribosome-bound EF-G adopts at least two different conformations that correspond to the pre- and posttranslocation states of the ribosome. Our results support a hypothesis suggesting that the movement of domain IV of EF-G plays a critical role in translocation.

Results

EF-G Conformation in Posttranslocation Ribosomes. The movement of EF-G relative to the A site of the small ribosomal subunit during translocation was followed using FRET between domain IV of EF-G and the ribosomal protein S12 (Fig. 1B). For EF-G labeling, a cysteine residue was introduced in domain IV of EF-G relative to the A site of the small ribosomal subunit using smFRET and total internal reflection microscopy. We show that domain IV of ribosome-bound EF-G adopts at least two different conformations that correspond to the pre- and posttranslocation states of the ribosome. Our results support a hypothesis suggesting that the movement of domain IV of EF-G plays a critical role in translocation.

Protein S12 is the only ribosomal protein located at the subunit interface on the body of the small subunit, near the 30S A site. A single cysteine mutant of S12 (L48C) was labeled with the donor (Cy3) dye and introduced into the 30S subunit using an in vitro reconstitution as previously described (34). A toeprinting translocation assay showed that, consistent with an earlier report (3), at least 50% of reconstituted ribosomes were able to form pretranslocation complexes and were active in translocation (Fig. S3). Nevertheless, the presence of inactive ribosomes does not affect our smFRET measurements because they are incapable of binding mRNA and becoming tethered to the microscope slide (Fig. 1C). We routinely checked for nonspecific binding of S12–Cy3-labeled ribosomes by adding reconstituted ribosomes to the slide in the absence of neutravidin and imaging the slide. Non-specific binding of reconstituted ribosomes was virtually absent.

Using smFRET, we first probed the most extensively characterized and well-defined conformation of ribosome-bound EF-G in which domain IV is docked into the 30S A site. A ribosome complex was assembled by nonenzymatic binding of N-acetyl-Met–tRNA⁹⁴⁴ to the P site of the E. coli ribosome containing a Cy3-labeled S12 in the presence of a defined mRNA followed by the addition of EF-Tu·GTP·Phe·tRNA⁹⁴⁴. The resulting pretranslocation complex, containing the dipeptidyl N-acetyl-Met–Phe·tRNA⁹⁴⁴ in the A site and deacylated tRNA⁹⁴⁴ in the P site was immobilized on a microscope slide/coverslip via a biotin-derivatized DNA oligonucleotide that was tethered via neutravidin to a microscope slide.
Recent cryo-EM structures of the ribosome revealed Salsi et al. – ce of the posttranslocation in the A site and deacy-

Representative smFRET trace for EF-G · www.pnas.org/cgi/doi/10.1073/pnas.1410873111 = C(16, 21, 38, 39). Thus, ribosomes with a vacant A site.

FRET pair used in our work is insensitive to intersubunit rotation in complexes was indistinguishable, indicating that the EF-G/S12

b o s o m e( 2 ,3 ,1 2 ,1 7 ,3 7 ) .H o w e v e r ,E F - G / S 1 2F R E Tint h e s et w o
tidyl-tRNA in the P site were previously shown to be predominantly

of EF-G is docked into the vacant A site of the small subunit (2, 24 (Fig. 3

in the ribosomes containing a single deacylated tRNA in the P site

measured between domain IV of EF-G (EF-G

domain IV of EF-G to the A site of the small subunit (24).

structure of posttranslocation ribosome that shows the binding of domain IV of EF-G to the A site of the small subunit (24).

A lone high (0.8) FRET state was also observed when FRET was measured between domain IV of EF-G (EF-G–S12-Cy5) and S12 in the ribosomes containing a single decylated tRNA in the P site (Fig. 3B) and the vacant A site. This result further supports as-

0.8 FRET state before the appearance of a short-lived (average dwell time, 400 ms) lower (∼0.6) FRET state without transition to the 0.8 FRET state cor-

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EF-G Adopts Different Conformations in Pre- and Posttranslocation Ribosomes. Recent cryo-EM structures of the ribosome revealed a previously unobserved conformation of EF-G, in which domain IV is bound outside of the A site of the small subunit. We next attempted to test whether this conformation is sampled by EF-G during translocation by flowing in EF-G–GTP–Fus to pre-

translocation ribosomes while imaging. However, real-time ob-

servation of translocation is hampered by the fact that the rate of translocation is determined to be 5–20 s⁻¹ (16, 21, 38, 39). Thus,

dwell time of EF-G in the pretranslocation state (τ = 1/κ) is expected to be 50–200 ms, which is near or below the time res-

olution of our smFRET measurements (100 ms). When EF-G–

Cy5–GTP–Fus was injected into the sample chamber during imaging of S12–Cy3 pretranslocation ribosomes containing
dipeptidyl N-acetyl-Met-Phe-tRNA³⁵ in the A site and decyl-

ated tRNA⁴³ in the P site, EF-G arrival was observed by bursts of Cy5 fluorescence. The 0.8 FRET state was predominantly ob-

served in smFRET traces. However, ∼43% traces showed sampling of a short-lived (average dwell time, 400 ms) lower (∼0.5–0.6) FRET state before the appearance of the posttranslocation 0.8 FRET state (Fig. S4A). The apparent 0.5–0.6 FRET state may correspond to the conformation that EF-G adopts in the pre-

translocation ribosome. Hence, observed sampling of a short-lived 0.5–0.6 FRET state without transition to the 0.8 FRET state corre-

responds to the arrival and rapid departure of EF-G and suggests that not all EF-G binding events result in productive translocation. Indeed, the transition to the posttranslocation conformation, indicated by the appearance of the 0.8 FRET state, required 1.5 EF-G binding events on average. This is consistent with recent single-molecule experiments reporting that translocation requires 1.6 EF-G binding events on average (18). Consistent with the assignment of the 0.5–0.6 FRET state to the conformation of EF-G bound to pretranslocation ribosomes, a small number of traces (less than 1%) show a detectable transition from the ∼0.5 to the 0.8 FRET state (Fig. S4 B and C). Hence, transitions from the ∼0.5 to the 0.8 FRET state may correspond to EF-G binding to the pretranslocation ribosome followed by translocation coupled with the movement of domain IV of EF-G into the posttranslocation conformation. This transition is likely not detected in a majority of traces because of the 100-ms time resolution limit of our smFRET experiments.

To further probe the pretranslocation conformation of the EF-

G–ribosome complex by extending its life time, the pretranslocation ribosome containing the dipeptidyl tRNA in the A site and decacy-

lated tRNA in the P site was preincubated with the antibiotic viomycin before the addition of fluorescently labeled EF-G. Viomycin strongly inhibits translocation but does not interfere with EF-G binding to the ribosome or GTP hydrolysis by EF-G (40, 41), and it also stabilizes the hybrid-state conformation of the ribosome (42). When EF-G–Cy5–GTP–Fus (less than 100 FRET states) was added to pretranslocation S12–Cy3 labeled ribosomes preincubated with viomycin before the beginning of imaging, in addition to the 0.8 FRET state, a second peak centered at 0.55 was observed in the FRET distribution histogram (Fig. 3C) built from smFRET traces (Fig. S5A). To examine whether the 0.55 FRET state corresponds to the pretranslocation ribosome, we performed the toeprinting translocation assay under experimental conditions (i.e., temperature and buffer conditions) similar to those in the smFRET experiments. The translocation assay shows that trans-

location occurs in about half of the ribosomes preincubated with viomycin, whereas the other half remain in the pretranslocation state (Fig. S6). A similar distribution between the high (0.8 FRET, 35%) and lower FRET state (0.55 FRET, 65%) supports the idea that the high (0.8) and lower (0.55) FRET states correspond to the post- and pretranslocation conformations of the EF-

G–ribosome complex, respectively.

When smFRET experiments were performed in the presence of viomycin alone, EF-G remained bound to the ribosome for ∼10 s on average despite the absence of Fus. This is consistent with data suggesting that viomycin inhibits EF-G dissociation from the ribosome after GTP hydrolysis (40). Compared with experiments performed in the presence of both Fus and viomy-

ycin, in the presence of viomycin alone the fraction of the post-

translocation conformation (0.8 FRET) decreased from 35% to 20% (Fig. 3E), whereas the fraction of EF-G in the pre-

translocation (0.55 FRET) conformation increased from 65% to 80%. Toeprinting experiments showed that fusidic acid does not affect the extent of mRNA translocation in ribosomes preincubated with viomycin (Fig. S6). The relative abundance of FRET states corresponding to pre- and posttranslocation states of the EF-G–ribosome complex depends on two factors: (i) the extent of translocation in the ribosome population and (ii) the stability of the EF-G–ribosome complex. Hence, the difference in the fraction of posttranslocation ribosomes between toe-

printing and smFRET experiments likely indicates that fusidic acid enhances EF-G binding to the posttranslocation ribosome (35), although having no effect on EF-G binding to the pre-

translocation ribosome (9).

When we reversed the dye-labeling scheme by attaching Cy3 to EF-G–S12–Cy5 and S12, the distribution between 0.6 (75%) and 0.8 (25%) FRET states in EF-G bound to pretranslocation ribosomes preincubated with viomycin (Fig. S7A) was similar to the one observed for the S12–Cy3/EF-G–S12–Cy5 FRET pair (Fig. 3E). Measurements of FRET between donor-labeled S12
(0.55 FRET) is directly induced by the binding of translocation inhibitors, i.e., the antibiotics viomycin or hygromycin B. Predominantly, the high (0.8) FRET state was observed for energy transfer between domain IV of EF-G (EF-G–541–Cy5) bound to ribosomes containing a deacylated tRNA$^\text{Met}$ in the P site and a vacant A site and preincubated with viomycin or hygromycin B (Fig. 3 D, F, and H). The presence of a small fraction (20%) of the low FRET state in the presence of viomycin might be due to the binding of noncognate tRNA$^\text{Met}$ to the A site in a small fraction of the ribosomes. This would not be surprising because viomycin is known to enhance A-site tRNA binding by 1,000-fold (41). Therefore, stabilization of the pretranslocation conformation of the EF-G–ribosome complex requires the presence of the A-site tRNA and is not directly induced by viomycin or hygromycin B. This interpretation is further supported by the fact that the FRET state corresponding to the pretranslocation conformation is observed, albeit rarely, in the absence of inhibitors of translocation (Fig. S4). The appearance of the low FRET state in the absence of viomycin or hygromycin B indicates that the pretranslocation conformation of the EF-G–ribosome complex is not an off-pathway state populated exclusively in the presence of antibiotics.

**Observed Movements of Domain IV of EF-G Are Not Directly Coupled to GTP Hydrolysis.** EF-G binding to the ribosome in the presence of viomycin and fusidic acid should allow for rapid GTP hydrolysis by EF-G. Moreover, viomycin does not affect P release (38), whereas fusidic acid produces a modest (approximately fourfold) decrease in single-turnover P release (43, 44). Because the rate of P release in the presence of fusidic acid and viomycin is 5 and 20 s$^{-1}$, respectively, GTP hydrolysis and subsequent P release are expected to be in the time scale of smFRET data acquisition. Thus, EF-G is likely bound to GDP or an apo-form in both FRET states observed in our smFRET experiments. To further explore the relationship between GTP hydrolysis and structural rearrangements of the EF-G–ribosome complex, we replaced GTP and Fus with a nonhydrolyzable analog of GTP, GDPNP. If the transition of EF-G from pre- to posttranslocation conformation is directly coupled to GTP hydrolysis then the replacement of GTP with GDPNP should result in stabilization of the 0.55 FRET state. However, when EF-G–541–Cy5 GDPNP was added to pretranslocation S12–Cy3-labeled ribosomes in the absence of translocation inhibitors, only a 0.8 FRET state was observed in FRET distribution histogram (Fig. 4 A). This result suggests that, consistent with published biochemical data (15), EF-G–GDPNP induces translocation and remains bound to the ribosome in the posttranslocation conformation corresponding to the 0.8 FRET state. When the pretranslocation complex was preincubated with viomycin or hygromycin B, the 0.55 and 0.8 states were observed in proportions similar to those observed in the presence of GTP and Fus (Fig. 4 B and C), thus suggesting that GTP hydrolysis is not directly coupled to observed structural rearrangements of the EF-G–ribosome complex.

Because the chemical properties of GDPNP are not identical to those of GTP (e.g., GDPNP binds to EF-G with significantly lower affinity) (45), we took an alternative approach by using a GTPase-deficient variant of EF-G to test the role of GTP hydrolysis in the movement of domain IV of EF-G. A conserved histidine (H92 in *E. coli* EF-G) of the switch loop II, which was proposed to play an important role in the catalysis of GTP hydrolysis by ribosome-activated GTPases such as EF-Tu and EF-G (46–48), was replaced with alanine. Consistent with recently published reports (47, 48), we found that the H92A mutation introduced into wild-type EF-G decreased the GTPase activity of EF-G by at least 100-fold (Fig. S8 A). Nevertheless, under single-turnover conditions (i.e., excess of EF-G over ribosome), EF-G (H92A) promoted the translocation of mRNA in toeprinting assays, indicating that EF-G (H92A) retains translocation activity (Fig. S8 B and Table S1). The H92A mutation was also

![Fig. 3. Histograms showing distribution of FRET values in different EF-G-ribosome complexes.](image-url)

The histograms show the distribution of FRET values in different EF-G-ribosome complexes. EF-G–541–Cy5-GTP was incubated with S12–Cy3-labeled pretranslocation ribosomes containing tRNA$^\text{Met}$ in the P site and N-acetyl-Met–Phen–tRNA$^\text{Met}$ in the A site (A, C, E, and G) or ribosomes containing a deacylated tRNA$^\text{Met}$ in the P site and vacant A site (B, D, F, and H). Experiments were performed in the presence of fusidic acid (A–D and G and H), viomycin (C–F), or hygromycin B (G and H). N is the number of EF-G binding events in single-molecule traces compiled for each histogram. Red lines represent Gaussian fits; the black line represents the sum of two Gaussians.
introduced into the single-cysteine variant of EF-G (S41C) and the resulting EF-G variant was labeled with an acceptor fluorophore. When EF-G (H92A)–Cy5·GTP was added to pretranslocation S12–Cy3-labeled ribosomes in the absence of translocation inhibitors, only the 0.8 (posttranslocation) FRET state was observed (Fig. 8C). When EF-G (H92A)–Cy5·GTP was added to pretranslocation S12–Cy3-labeled ribosomes preincubated with viomycin (Fig. S8D), the 0.55 and 0.8 FRET states were observed in proportions similar to the ones observed in wild-type EF-G in the presence of GTP and Fus (Fig. 3C). Thus, inhibition of GTP hydrolysis by the substitution H92A stabilizes EF-G binding to the posttranslocation ribosomes similar to Fus (Fig. S8D) as also evident from comparison between FRET distribution histograms for wild type and H92A variants of EF-G obtained in the presence of viomycin and the absence of Fus (Fig. 3E and Fig. S8D). In conclusion, inhibition of GTP hydrolysis did not result in the stabilization of the pretranslocation (0.55) FRET state, suggesting that the transition from the pre- to the posttranslocation conformation of domain IV of EF-G is not coupled to GTP hydrolysis.

Discussion

Using FRET between fluorophores attached to EF-G and ribosomal protein S12, we directly followed structural rearrangements of domain IV of ribosome-bound EF-G in solution. Our data provide independent evidence that domain IV of EF-G undergoes a significant movement during translocation toward the A site of the small subunit. We observed a single (0.8) FRET state when EF-G was bound to the ribosome with a vacant A site. The 0.8 FRET state likely corresponds to the conformation of EF-G previously visualized by X-ray crystallography and cryo-EM structures of similar EF-G–ribosome complexes (2, 24, 26–28), in which domain IV of EF-G is docked into the 30S A site. When EF-G was bound to pretranslocation ribosomes containing tRNAs in both the A and P sites in the presence of an inhibitor of translocation, viomycin or hygromycin B, an additional 0.55 peak was observed in FRET distribution histograms (Figs. 3, 4, and Fig. S8). Lower (0.55) FRET value indicates that in the pretranslocation conformation, domain IV is positioned farther away from protein S12 and placed on the outside of the A site of the small ribosomal subunit, which is occupied by the A-site tRNA. This finding is consistent with cryo-EM reconstruction of EF-G–ribosome complexes trapped in the pretranslocation state in the presence of viomycin (9), in which domain IV of EF-G is positioned outside of the 30S A site and contacts the A site tRNA.

Notably, the 0.55 peak was consistently ~1.5-fold wider than the 0.8 peak in all FRET distribution histograms. The width of each peak is defined by a combination of signal-to-noise ratio and potential conformational heterogeneity. Hence, the relatively wide width of the 0.55 FRET peak might indicate the presence of structural heterogeneity in the pretranslocation conformation of EF-G. Indeed, if we assume that signal-to-noise ratio and the peak widths are the same for equally represented FRET states, then the histogram for the pretranslocation EF-G–ribosome–viomycin complex is best fit to three Gaussian distributions with self-consistent widths (Fig. S9). Thus, the broad 0.55 peak might be a sum of two FRET peaks centered at 0.5 and 0.6 FRET values (Fig. S9). A limited number of traces showing transitions (less than 5% of all traces) hampered further testing of the three-state model using hidden Markov modeling or other algorithms used for the identification of defined FRET states in single-molecule traces. Nevertheless, the three-state fitting of FRET distribution histograms (Fig. S9) suggests that domain IV might adopt more than one conformation before translocation. Further structural studies are required to verify this hypothesis.

Interestingly, toeprinting and smFRET experiments suggested that EF-G·GTP induces translocation in a large fraction of ribosomes even in the presence of viomycin and hygromycin B, which were reported to inhibit the rate of translocation by 10,000- and 300-fold, respectively (41). It is possible that at the time scale of smFRET and toeprinting experiments (in minutes), antibiotics may transiently dissociate from the ribosome allowing EF-G to catalyze translocation. In addition to transitions from the low (pretranslocation) to high (posttranslocation) FRET state (Fig. S5F), rare transitions from the high to low FRET state were also observed in two tRNA ribosome complexes incubated with viomycin (Fig. S5C). These transitions may correspond to spontaneous sampling of the pretranslocation conformation of EF-G in posttranslocation ribosomes (i.e., ribosomes with a vacant A site). However, high-to-low FRET transitions may also correspond to reverse translocation of tRNAs on the small subunit. Reverse translocation was previously observed in vitro under certain experimental conditions and shown to be stimulated by viomycin (49, 50). Hence, the high-to-low FRET state transitions might be indicative of equilibrium between pre- and posttranslocation states of the EF-G–ribosome complex maintained by the presence of EF-G·GTP and translocation inhibitors.

Remarkably, replacing GTP with a nonhydrolyzable analog of GTP, GDPNP or assembling ribosomal complexes with a GTPase-deficient mutant of EF-G did not result in the stabilization of the 0.55 FRET state corresponding to the conformation adopted by EF-G before translocation. Thus, the movement of domain IV of EF-G toward the 30S A site during translocation does not seem to be directly coupled to GTP hydrolysis. Further kinetic studies may be required to validate this conclusion. It is important to note that our FRET pairs only follow the movement of domain IV of EF-G relative to the A site of the small subunit. Hence, GTP hydrolysis may trigger other structural rearrangements of EF-G not detected in our work. In particular, several lines of evidence indicate that rearrangements of switch loops I and II in the G domain of EF-G are induced by GTP hydrolysis and Pi release and are critical for EF-G dissociation from the ribosome (26–28, 51, 52).

Our smFRET experiments support the idea that the movement of domain IV of EF-G toward the A site of the small subunit promotes the translocation of peptidyl tRNA from the A into the P site. These smFRET experiments provide important clues about the relative stability of the alternative conformations of the EF-G–ribosome complex. EF-G bound to a ribosome with a vacant A site displayed a single 0.8 FRET state regardless of whether the P site was occupied with peptidyl (Fig. 3A) or deacylated tRNA (Fig. 3B) (i.e., regardless of whether the ribosome was stabilized in a nonrotated or rotated conformation,
respectively), and transitions into the 0.55 FRET state were extremely rare. The 0.55 FRET state could be stabilized only in the presence of translocation inhibitors and A-site tRNA, which precluded the movement of domain IV into the 30S A site. These observations suggest that the conformation of the EF-G–ribosome complex corresponding to the 0.55 FRET state, in which domain IV of EF-G is positioned outside of the 30S A site, is much less stable than the conformation of EF-G corresponding to the 0.8 FRET state, in which domain IV of EF-G is docked into the 30S A site. Therefore, the translocation of tRNAs on the small ribosomal subunit appears to be coupled to the transition from a high-energy state to a more stable conformation of the EF-G–ribosome complex. Thus, the structural rearrangement of EF-G on the ribosome makes a considerable energetic contribution to promoting tRNA translocation.

Materials and Methods

Materials and Methods are described in detail in SI Materials and Methods. The mRNA m291, ribosomes, EF-Tu, aminocaylated tRNAs, and reconstituted ribosomes were prepared as previously described (3, 53, 54). Single-molecule fluorescence measurements were obtained using a prism-type total internal reflection (TIR) microscope as described (17). Apparent FRET efficiencies ($\epsilon_{\text{app}}$) were calculated from the emission intensities of donor (I donor) and acceptor (I acceptor) as follows: $\epsilon_{\text{app}} = \frac{I_{\text{donor}}}{I_{\text{donor}} + I_{\text{acceptor}}}$. Acknowledgments: We thank Harry Noller for providing plasmids containing constructs EF-G–cys, EF-G–G4TC, and S12–G4BC; Farzaneh Todnevis for her early contribution to the project; Peter Cornish for sharing Matlab scripts and advice on TIR microscopy; and Harry Noller, Gloria Culver, Andrei Korostelev, Paul Whittford, and Peter Cornish for helpful discussions. These studies were supported by the National Institutes of Health Grant GM–090719 (to P.E.N.) and Grant P30 GM–092424 (to the Center for RNA Biology, University of Rochester).