

Self-Association of Enolase from *Trichomonas vaginalis*. Monomers, Dimers, and Octamers Coexist in Solution

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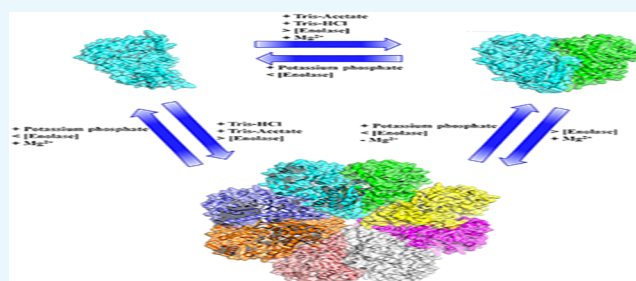
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Supporting Information

ABSTRACT: We used small-angle X-ray scattering to study the self-association of enolase from *Trichomonas vaginalis* as a function of the protein concentration and cosolute type. We observed coexisting monomers, dimers, and octamers in variable relative populations, depending on whether Tris-acetate, Tris-HCl, or potassium phosphate buffers were used. Phosphate ions hindered the formation of dimers and octamers. In contrast, the populations of dimers and octamers increased in Tris-acetate or Tris-HCl buffers and additionally increased by augmenting protein concentration or adding magnesium. Single oligomeric species could not be isolated in any of the experimental conditions tested. Furthermore, the secondary and tertiary structures, as well as the temperature-induced denaturation of the mixtures of species, were investigated. The acquired species lost enzymatic activity, but they were prone to interact with plasminogen, as judged from changes in the secondary and tertiary structures upon complex formation.



INTRODUCTION

Enolase is a moonlighting protein that performs a variety of functions. Its canonical task is catalyzing the reversible dehydration of 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate (PEP) in glycolysis and gluconeogenesis. For catalysis, Mg^{2+} is the main physiological cofactor of enolase and promotes its highest activity but some other metals, such as Mn^{2+} and Zn^{2+} , can also bind and activate the enzyme.¹ Enolase is also involved in cellular stress; bacterial, parasitic, and fungal infections; and autoantigen activities, as well as the occurrence and metastasis of cancer and the growth, development, and reproduction of organisms.² Markedly, the surface localization of enolase has been shown for several cell types, including bacteria, fungi, protozoans, and mammals, serving to recruit and promote plasminogen activation pericellularly and contributing to immune evasion and tissue invasion processes.^{3–6}

Structurally, enolase is typically a homodimeric protein in which each monomer has a large domain (C-terminal) with an $\alpha\alpha\beta\beta$ (α/β)₆ barrel folding type and a small domain (N-terminal) with three α -helices and four β -strands. The active

site is located at one end of the barrel, but both domains include loops that fold over the active site when the substrate is bound.^{7–10} Subunit contacts for homodimer formation are between the small domain of one subunit and the large domain of the other. In addition, the crystal structure of enolase from some species, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus suis*, and *Bacillus subtilis*, has revealed octameric arrangements,^{10–13} which are described as wheel-shaped scaffolds formed from a tetramer of dimers.¹⁴ In octamers, the overall scaffolds of monomers and dimers are quite similar to those of dimeric enolases. The octameric structure holds two types of oligomerization interfaces opposite to each other: one holds the two monomers together (to form dimers), and the other corresponds to neighboring dimers (to form octamers).¹⁵

Trichomonas vaginalis is a protist that causes the most common nonviral sexually transmitted infection in humans,

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known as trichomoniasis.¹⁶ In the *T. vaginalis* sequence database,¹⁷ there are nine genes annotated as enolase, even though at least two of them should be incapable of performing enzymatic activity since they lack the active-site amino acid residues. Furthermore, the product of the gene encoded by TVAG_329460 (UniProt entry A2EBB4) was described as an anchorless, surface-associated plasminogen receptor in *T. vaginalis*.¹⁸ In addition, enolases from *T. vaginalis* show two distinctive characteristics from the enolase of the human host. One characteristic corresponds to three key substitutions within one of the loops of the active site compared with host enolase. The other characteristic is a unique N-terminal motif, composed of 15–18 residues, on all potentially active enolases, whose function still must be established. These features have positioned enolase as an important cell-surface virulence factor and as a potential drug and vaccine target as well as a diagnostic marker to fight trichomoniasis.¹⁹

The crystallographic structure has not been solved for any of the enolases from *T. vaginalis*, and the oligomeric state of them is yet to be analyzed. Therefore, we studied the oligomeric state of enolase from *T. vaginalis* (TvEno) in a variety of experimental conditions. We found that monomers, dimers, and octamers coincide in solution and their populations depend on the cosolute and protein concentrations. The secondary structure and tryptophan environment as well as thermal unfolding transitions of enolase solutions were studied by circular dichroism (CD) and fluorescence spectroscopies.

Enolase is a moonlighting protein, which participates in multiple biochemical processes, apart from enzymatic activity, in several cellular locations. This means that enolase is submitted to a variety of physicochemical environments. One of the most studied nonenzymatic functions of enolase is binding of plasminogen on the cell surface of several organisms. Some reports indicate that non-native structures (monomers and multimers) of *Streptococcus pyogenes* (Sp) enolase are capable of interacting with canine plasminogen. On the contrary, when Sp enolase and plasminogen are soluble, correctly and completely folded, and in the case of enolase, fully active, no binding occurs.⁵⁵ Therefore, a bunch of questions arise; for example, what gives enolase its functional plasticity? What is the conformation or oligomeric state that performs each of the jobs of enolase? How enolase is targeted to multiple cellular locations? What determines the specificity of enolase for each of its ligands? Since information about the structure of the enolase under the various experimental conditions is lacking, we were curious to investigate the oligomeric state, secondary and tertiary structures, and stability of enolase in a variety of physicochemical conditions, particularly where enolase does not show enzymatic activity. Indeed, these conditions are not identical to those found in the cellular environment but our results shed light on the conformational and oligomeric-state changes that enolase can undergo in performing a variety of functions, particularly plasminogen binding.

RESULTS

Purification and Enzymatic Characterization. All enolases from *T. vaginalis* have a unique N-terminal extension of 34–63 amino acid residues, which is absent in enolases from other organisms.¹⁹ In this work, an N-terminal truncated version of enolase, eliminating 34 amino acid residues (TvEno Δ 34), was used in all experiments. This construct is similar in the number of amino acid residues in enolases from

other biological species. The recombinant His-tag-cleaved enolase protein was successfully purified, producing yields of approximately 7.5–10 mg of protein per liter of culture. Kinetic characterization of the TvEno Δ 34 enolase construct was successfully achieved in triethanolamine (TEA) buffer. The kinetic parameters determined are as follows: specific activity of 212.36 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ of enzyme, a turnover (k_{cat}) of 3.53 s^{-1} , and a K_{M} equal to 5.17 nM. Several attempts to measure the enzymatic activity in Tris–HCl, Tris–acetate, or potassium phosphate buffers were made under a variety of experimental conditions (different temperatures were tested as well as concentration of reactants). Nevertheless, we could not detect the conversion of the substrate 2-PGA into the product PEP when buffers different from TEA were used. Conversely, enzymatic activity was detectable in positive control assays, using enolase from *Saccharomyces cerevisiae*, in Tris–acetate and Tris–HCl buffers, with similar specific activity as in TEA buffer (110 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ of enzyme). As discussed below, slight conformational changes (involving secondary and tertiary structures) might have occurred to enolase from *T. vaginalis* when it was diluted in Tris–acetate and in Tris–HCl buffers. Consequently, the fine arrangement of amino acid residues and loops within the catalytic site on the protozoan enzyme was altered. Regarding the phosphate ions, they are a well-known competitive inhibitor of enolase.¹ As expected, we could not detect enzymatic conversion for either TvEno Δ 34 or enolase from yeast.

Oligomeric State of Enolase Solutions. Small-angle X-ray scattering (SAXS) is useful for determining the oligomeric states and quaternary structures of proteins in solution.²⁸ The SAXS Kratky profiles (Figure S1) allowed us to determine the size and shape of enolase solutions under a variety of experimental conditions. The size distributions obtained are summarized in Figure 1. In most buffers, at least two protein

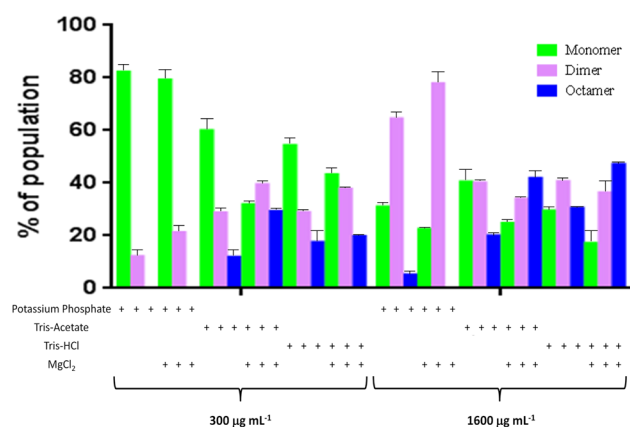


Figure 1. Percentage of population of each oligomeric state of TvEno Δ 34 from *T. vaginalis* in different buffer conditions. SAXS experiments were performed using two different protein concentrations (300 and 1600 $\mu\text{g mL}^{-1}$, as indicated). The buffer solution in which enolase was diluted is indicated with crosses at the bottom of the figure.

concentrations were evaluated (300 and 1600 $\mu\text{g mL}^{-1}$) and in some cases, protein solutions at 30 $\mu\text{g mL}^{-1}$ were also tested. The most remarkable feature of this data is that monomers, dimers, and octamers could be detected in equilibrium, in most of the experimental conditions, depending on the protein concentration and the nature of the cosolute. In all conditions

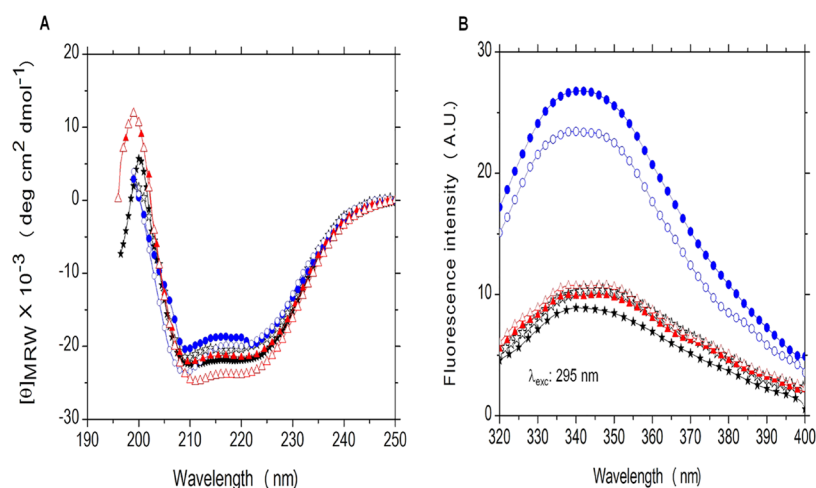


Figure 2. (A) Far-UV circular dichroism and (B) intrinsic fluorescence-emission (excitation wavelength 295 nm) spectra of native recombinant TvEno Δ 34. Enolase was diluted in Tris-HCl buffer (solid stars), Tris-HCl buffer complemented MgCl₂ (open stars), Tris-acetate buffer (solid circles), Tris-acetate buffer plus MgCl₂ (open circles), potassium phosphate buffer (solid triangles), and potassium phosphate buffer with MgCl₂ (open triangles). All spectra were obtained at 25 °C and pH 8.0. The final concentration of MgCl₂ was 2 mM.

tested, it was not possible to obtain monodisperse systems, with a single species populated, even by varying protein concentrations by more than 50-fold.

Additionally, some attempts to isolate a single oligomeric-state species by gel-filtration chromatography were made (Agilent Bio SEC-5, HPLC column, 0.2 mL min⁻¹; data not shown) but equilibria were rapidly restored, according to protein concentration and cosolute. In the following paragraphs, we describe the results obtained in each buffer solution.

Potassium Phosphate Buffer. The highest proportion of monomers was detected in potassium phosphate buffer at 30 (data not shown) and 300 μ g mL⁻¹ either in the absence or presence of Mg²⁺ (\approx 82 and 79%, for apo- and holo-enolase, respectively). When the protein concentration was increased up to 1600 μ g mL⁻¹, using this buffer, the percentage of dimers increased (64.3 and 77.7% for apo- and holo-enolase, respectively). Interestingly, only a small amount of octamers could be detected but only in the case of apo-enolase (6.1 and 4.9% at 300 and 1600 μ g mL⁻¹, respectively; octamers were not detected at the lowest protein concentration of 30 μ g mL⁻¹) since octamers could not be observed when the divalent cofactor was added to the protein solution.

Tris-HCl Buffer. As expected, the oligomerization state was highly dependent on the protein concentration. In this buffer solution, the monomer dominated at a low protein concentration (300 μ g mL⁻¹) for both apo- (54.1%) and holo-enolase (43%). Under these conditions, the dimer constituted 28.6 and 37.5% of the protein solution in the absence and presence of Mg²⁺, respectively. The octamer accounted for 17.3 and 19.5% for apo- and holo-enolase, respectively. When the protein concentration was increased to 1600 μ g mL⁻¹ in Tris-HCl buffer, monomers were less abundant and the concentration of dimers and octamers increased. For apo-enolase, at 1600 μ g mL⁻¹, monomers and octamers were almost equally populated (\approx 30% of each species) whereas the population of the dimer accounted for approximately 40%. In the case of holo-enolase, the octamer was even more favored (46.9%; the highest concentration of octamers obtained in all conditions tested) whereas the population of monomers dropped to 16.9% and the dimer was slightly increased to 36.2%.

Tris-Acetate Buffer. At low protein concentrations (300 μ g mL⁻¹), monomers were favored for apo-enolase (59.8%), followed by dimers (28.5%), whereas octamers represented 11.7% of the population of this solution. When the divalent cofactor was added to the solution while maintaining the protein concentration, dimers were favored (39.3%), followed by monomers in which the concentration dropped to 31.7% and the octamer population increased to 29%.

At high protein concentrations of apo-enolase (1600 μ g mL⁻¹), monomers and dimers were almost equally populated (\approx 40%) whereas octamers represented approximately 20% of the species. At this protein concentration, for holo-enolase, the highest order oligomeric state was preferred (41.7%). In this case, the monomer and dimer populations were 24.5 and 33.8%, respectively.

Secondary and Tertiary Structures of Monomer-Dimer-Octamer Mixtures. The secondary structure and hydrophobic environment of tryptophanyl residues of the mixtures with variable populations of monomer-dimer-octamer were assessed by circular dichroism and intrinsic fluorescence spectroscopies. Since all solutions studied represent at least two different oligomeric states, the CD and fluorescence signals are the contribution of all species present in each solution, according to their relative populations.

In Figure 2, the CD and intrinsic fluorescence-emission spectra of apo- and holo-enolase at three different buffer conditions, 50 mM potassium phosphate, 50 mM Tris-acetate, and 50 mM Tris-HCl, at pH 8 are shown. In all cases, the concentration of MgCl₂ was 2 mM (although other concentrations of MgCl₂ were also evaluated, the data are not shown). All CD spectra were not identical but quite similar, showing the characteristic shape of an α/β protein, with two negative peaks centered at approximately 220 and 208 nm. The largest CD signal intensity was observed for enolase diluted in potassium phosphate buffer, complemented with Mg²⁺, the buffer condition where the relative population of monomers was close to 80%, dimers represented approximately 20% of the total protein, and octamers were nearly absent. CD spectra obtained in other buffer conditions showed that the helical content of enolase might decrease while increasing the β -sheet content. Considering the relative populations of monomers,

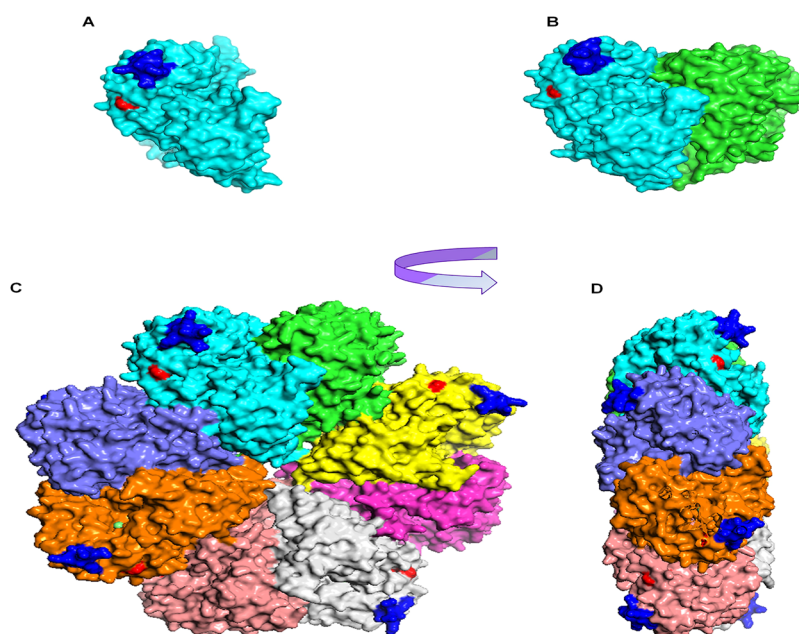


Figure 3. Three-dimensional models of monomers, dimers, and octamers of TvEno Δ 34. The subunits of each structure are colored for clarity. In each panel, Trp314 and 348 are represented in red. The putative plasminogen-binding site is shown in blue. (A) Monomer, (B) dimer, and (C, D) octamer.

dimers, and octamers in each buffer condition, it appears that an increase in dimer concentration and/or formation of the octameric species led to a slight decrease in helical content with a concomitant increase in β -sheet content.

Markedly, the effect on the secondary structure of the divalent cation was different in each buffer solution. In Tris–HCl buffer complemented with 2 mM MgCl₂ (where dimers and octamers were increased in relative population), TvEno Δ 34 showed less helical content than in the absence of the cofactor. In contrast, when Tris–acetate buffer was complemented with the same MgCl₂ concentration and concomitantly, dimers and octamers were favored with respect to monomers, enolase gained helical structure (Figure 2). This means that different cosolutes apart from leading to different combinations of oligomeric species might also induce slightly different conformations of these species.

Intrinsic fluorescence-emission spectra ($\lambda_{\text{exc}} = 295$ nm) are shown in Figure 2. The values of the corresponding spectral center of mass (SCM) are shown in Figure S2. These values varied between 352 and 355 nm depending on the buffer solution used. The shortest SCM value (352 nm) was observed for holo-enolase in Tris–acetate buffer, whereas the largest SCM observed corresponded to holo-enolase diluted in Tris–HCl buffer. Furthermore, fluorescence spectra showed different emission intensities; the highest fluorescence-emission intensities were detected for enolase diluted in Tris–HCl buffer, either in the absence or presence of MgCl₂. TvEno Δ 34 showed a similar fluorescence-emission intensity when it was diluted either in Tris–acetate buffer or potassium phosphate buffer. The main differences between these spectra were the positions of the SCM.

These results confirm slight variations in the conformation of enolase from *T. vaginalis* when changing buffer solutions. In Figure 3, the three-dimensional (3D) models corresponding to monomeric, dimeric, and octameric TvEno are shown. In the three models, the structures of monomers are almost superimposable, except for some displacements of loops and

markedly of helix α 1 situated at the dimer–dimer interface when the octamer is formed. Additionally, β -strand 3, at the monomer–monomer interface seems to be shorter (adopting a more relaxed conformation) in the monomer than in the dimer or the octamer.

Regarding tryptophan residues, the sequence of TvEno has two of these, which are situated in helix α 10 (Trp314) and in the loop α 11 and β -strand 11 (Trp348). They appear as close neighbors in the three-dimensional structure. Since Trp348 is in a loop, it might have higher mobility and exposure to the solvent than Trp314. Unpublished data from our lab obtained by molecular dynamics simulation of monomeric enolase confirm that the side chain of Trp348 might have a larger tendency to be exposed to solvent than Trp314. Neither Trp314 nor Trp348 are near the interfaces (monomer–monomer or dimer–dimer). Therefore, these residues cannot participate in interface formation. This means that changes in the SCM of the fluorescence-emission spectra of TvEno Δ 34 due to modification of the buffer solution were a consequence of conformational adaptations within each monomer when forming different oligomeric-state species.

Thermal Stability of Monomer–Dimer–Octamer Mixtures. The thermal denaturation of *T. vaginalis* enolase solutions was assessed by recording the temperature-induced denaturation profiles in potassium phosphate, Tris–HCl, or Tris–acetate buffers. The transitions were followed by monitoring the ellipticity at 220 nm, with constant heating rates (2 °C min^{−1}), as shown in Figure 4. We observed that transitions obtained in Tris–acetate buffers without Mg²⁺ appear as single sigmoid curves, whereas the transition in Tris–HCl buffer without Mg²⁺ appeared as a very broad, complex sigmoid curve, indicating the presence of several steps between 35 and 85 °C. The profile obtained in potassium phosphate buffer shows an interesting characteristic. The secondary structure of apo- and holo-enolase was not significantly altered by thermal treatment in potassium phosphate buffer conditions, as judged from the CD signal,

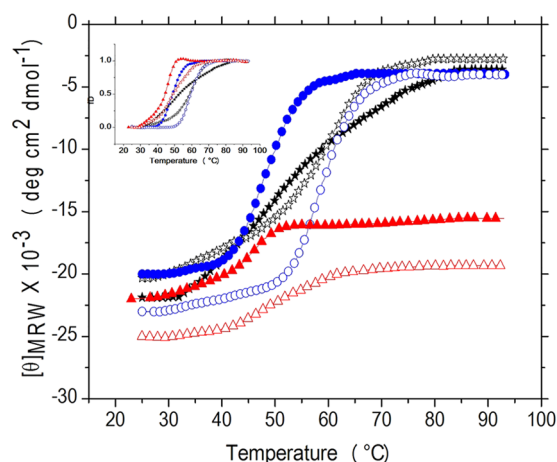


Figure 4. Temperature-induced thermal denaturation profiles of enolase TvEno Δ 34 monitored by CD spectroscopy in Tris-HCl buffer (solid stars), Tris-HCl buffer plus MgCl₂ (open stars), Tris-acetate buffer (solid circles), Tris-acetate buffer complemented with MgCl₂ (open circles), potassium phosphate buffer (solid triangles), and potassium phosphate buffer complemented MgCl₂ (open triangles). The denaturation process was followed by recording ellipticity at 220 nm. The inset shows normalized data. The final concentration of MgCl₂ was 2 mM.

which was reduced by approximately 5 units (θ_{MRW}), whereas profiles obtained in other buffer solutions had a reduction of the signal of approximately 15–18 units (θ_{MRW}). This means that the unfolded state obtained in potassium phosphate buffer retained a considerable amount of secondary structure and might be more compact, compared with the unfolded state achieved in the other two buffer solutions (either for apo- or holo-enolase).

When protein solutions were complemented with MgCl₂ (2 mM), the thermal denaturation process occurred at higher temperatures compared to those for protein solutions without MgCl₂ (Figure 4). This was more easily noticeable when the transitions were normalized (Figure 3 inset). This means that the divalent cofactor stabilized enolase from *T. vaginalis*, as observed for enolase from other biological species²⁹ as well as other enzymes that are stabilized by their cofactors.^{30,31} In the case of TvEno, subunit interactions necessary to increase the percentage of dimers and oligomers as observed in Tris-HCl and Tris-acetate buffers upon cofactor binding might have contributed to the observed thermal stabilization (Figure 4). Commonly, from thermal denaturation profiles, it is possible to obtain thermodynamic parameters associated with the unfolding or refolding reaction when a denaturation model can be assumed to describe the experimental data. In this case, as stated before, enolase solutions were mixtures of oligomeric species; therefore, it was not possible to establish a denaturation model that can adequately describe the profiles.

Interaction with Plasminogen. CD is a widely used method to determine the secondary structure of proteins in solution. It, thus, can be used to determine whether there are changes in protein conformation when proteins interact with each other.^{32–34}

The possible binding of plasminogen to the mixtures of TvEno Δ 34 and the conformational changes associated with this binding were analyzed using intrinsic fluorescence and far-UV CD. The spectra were recorded for each isolated protein and for both proteins in combination and are shown in Figure

5. In the same figure, the subtraction of the spectra is the spectra of the combined proteins minus the spectra of each protein recorded individually. In this case, four possible scenarios can be observed. The simplest one is that the TvEno Δ 34 mixture does not interact with plasminogen; therefore, CD and fluorescence spectra should remain unaltered and the spectra of the combination (plasminogen + TvEno Δ 34) must be equivalent to the algebraic addition of the spectra recorded for each of the proteins. A second possibility is that plasminogen and TvEno Δ 34 do interact, but this interaction does not modify the secondary or tertiary structures of either of the proteins involved. In this case, the complex formations should be transparent to CD and fluorescence spectroscopies. This case would not be distinguishable from the first scenario. Another option is that the interaction of plasminogen and TvEno Δ 34 occurs while altering the secondary and/or tertiary structure of only one of the proteins. In that case, the CD and/or fluorescence spectra would be different only for one of the proteins, with appreciable changes in the spectroscopic signals of the other protein upon complex formation. The last scenario would imply complex formation with concomitant conformational changes in both proteins that are appreciable in the CD and/or emission fluorescence signals.

Figure 5 shows a series of CD and fluorescence-emission spectra of TvEno Δ 34 and plasminogen in each of the buffers studied here in the absence and presence of MgCl₂. The results are also described for each buffer. It is important to mention that the plasminogen-binding site of enolase from *S. pneumoniae* is currently the best characterized. Two sites have been set; one of them is known as the C-terminal site and is composed of two lysine residues (⁴³³KK⁴³⁴) at the C-terminus. This site is nonconserved in enolases from *T. vaginalis* and in several confirmed plasminogen-binding enolases from several biological species. The internal site is composed of a nine-residue sequence motif (²⁴⁸FYDKERKVV²⁵⁶). Considering the sequence and structural alignments of enolases from *S. pneumoniae* and the product of gene TVAG_329460, the putative plasminogen-binding motif in TvEno Δ 34 corresponds to ²⁶⁴FYDEEKLY²⁷² (Figures 3 and S3).

Tris-HCl Buffer. In the absence of a divalent cofactor, there were no significant changes in the secondary structure upon combining plasminogen and TvEno Δ 34 since the CD signal of the mixture was almost equivalent to the addition of the spectra of each protein recorded separately. In contrast, the fluorescence signal of enolase was importantly quenched by the addition of plasminogen. The emission of the plasminogen was slightly quenched due to interaction with TvEno Δ 34. It is important to note that both tryptophan residues in TvEno Δ 34 are close to the putative plasminogen-binding site, so it is plausible that the interaction of enolase with plasminogen would modify the hydrophobic environment of the tryptophans.

When Mg²⁺ was added to this solution, we observed changes in the secondary structure of both enolase and plasminogen. The fluorescence signal of enolase was completely quenched. Since the addition of divalent cofactor implies an increment in dimer (mainly) and octamer concentrations, it implies that enolase might have a higher affinity toward plasminogen, as judged for larger changes in both spectroscopic signals, compared to that the solution without Mg²⁺. Notably, not only was enolase modified in its secondary and tertiary structures but also plasminogen adopted a different secondary

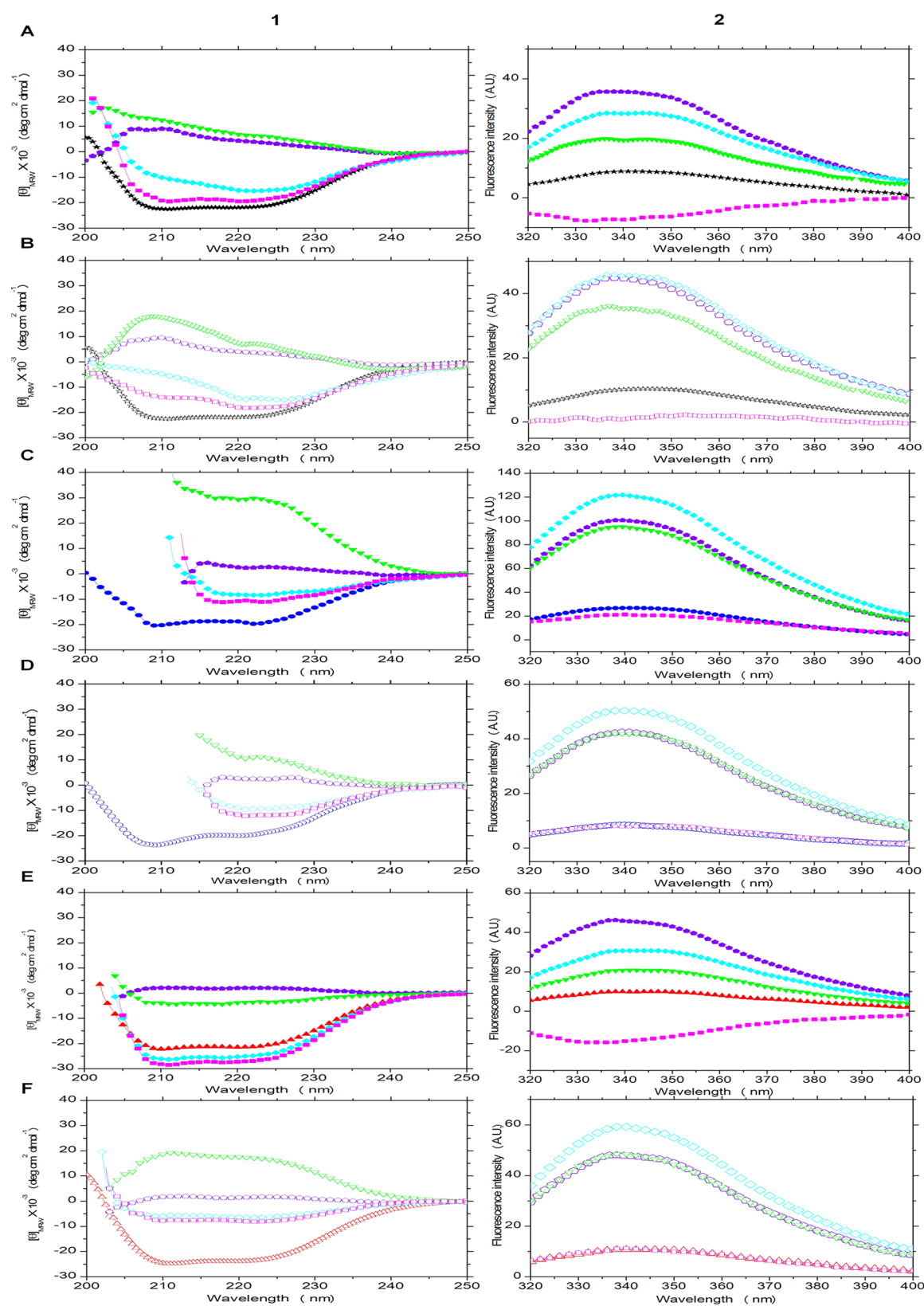


Figure 5. CD (1) and fluorescence-emission (2) spectra of different mixtures of enolase TvEno Δ 34 complexed with human plasminogen. Experiments were performed in native conditions at 25 °C and pH 8 in (A1, A2) Tris-HCl buffer; (B1, B2) Tris-HCl buffer complemented with MgCl₂; (C1, C2) Tris-acetate buffer; (D1, D2) Tris-acetate buffer with MgCl₂; (E1, E2) potassium phosphate buffer; and (F1, F2) potassium phosphate buffer plus MgCl₂. Samples are represented as follows: enolase (black stars, blue circles, and red triangles), plasminogen (violet pentagons), and enolase-plasminogen complex (cyan rhombus). The subtraction of the spectra corresponds to the spectra of the complex minus the corresponding spectra of native enolase (green triangles) or minus the spectra of plasminogen (magenta squares), which are also shown. Samples enriched with 2 mM MgCl₂ are represented with open symbols.

structure, as judged from the CD signal recorded in the absence and presence of Mg^{2+} in this buffer solution.

Tris–Acetate Buffer. In this buffer solution, either in the presence or absence of the divalent cofactor, we observed CD signal changes in both proteins but changes in plasminogen were more relevant. It seems that when the complex was formed, plasminogen gained secondary structure, whereas enolase lost it. Regarding the fluorescence-emission spectra, in both cases, the spectra of the combination corresponded merely to the addition of the individual contribution of the spectrum of each isolated protein. This means that despite important changes in the secondary structure of each protein upon interaction, they do not involve important changes in the hydrophobic environment of tryptophans. The changes in the spectra were similar in both conditions (with and without Mg^{2+}) but were more prominent in the absence of the cofactor.

Potassium Phosphate Buffer. In this solution, in the absence of $MgCl_2$, enolase gained secondary structure (α -helical content) upon complex formation, whereas the shape of the CD spectrum of plasminogen implied a transition between two random coil arrangements. Additionally, the fluorescence emission of both proteins was quenched upon interaction.

When the potassium phosphate buffer solution was complemented with Mg^{2+} and concomitantly, the percentage of dimers was raised from 11 to 20% and there was no population of detectable octamers, enolase lost an important amount of regular secondary structure whereas the CD signal of plasminogen indicated important changes in its secondary structure due to complex formation. In this case, the tryptophan environment of both partners was unchanged upon interaction.

DISCUSSION

Enolase is an enzyme that plays a central role in the carbon metabolism of most organisms by participating in glycolysis and gluconeogenesis. Enolase also participates in a variety of functions and has multiple locations either inside or outside cells. The functions might include transport,³⁵ cell adhesion,³⁶ actin cytoskeleton remodeling,³⁷ and plasminogen receptor^{18,19,38,39} among many other options, depending on the biological species. Nevertheless, it is not fully established whether the moonlighting functions of enolase are evolutionarily conserved. Neither the structure nor the oligomeric state necessary to perform each function is well defined. The physiological environment in which enolase is found may play an important role in its oligomeric state and consequently in its function.⁵ Therefore, this protein has become an important target for drug and vaccine designs, to fight either infections caused by bacteria⁴⁰ or parasites,⁴¹ or cancer,^{42–44} and even to cure diabetes.^{45,46} Therefore, describing the structure, the oligomeric state, and the mechanisms or conditions for self-association, which might be related to stability, multifunctionality, and trafficking within the cell among other characteristics of this protein, offers additional perspectives for both drug discovery and vaccination. Particularly, for enolases from *T. vaginalis*, this information has relevance due to the special sequence characteristics of these proteins, including the three key substitutions within one of the loops of the active site, compared to those of the human host enolase, as well as the unique N-terminal motif, composed of 15–18 residues, observed in enolases from the parasite.¹⁹

The ability of enolases from several biological species to self-associate and dissociate into monomers has been the subject of

several studies.^{47–54} In some cases, enolase can be detected merely in its dimeric state under native conditions^{55,56} and monomers can be obtained by submitting the enzyme to chaotropic agents, such as temperature and hydrostatic pressure, and chemical agents, such as sodium perchlorate, or imidazole.^{47–54} Whether or not the monomer of enolase has enzymatic activity depends on the biological origin of the enzyme and the conditions of dissociation. Additionally, some enolases can populate only the octameric state¹⁵ but mixtures of oligomeric-state species have also been reported.¹⁴ For example, the octameric enolase from *S. pyogenes* could be dissociated into monomers but dimers could not be produced, nor could any other oligomeric state.¹⁴

Here, we found three oligomeric-state species of a recombinant construct of enolase from *T. vaginalis* (TvEno Δ 34) coexisting in solution under nonharsh physicochemical conditions by changing the buffer solution in which enolase is diluted. The relative population of the species depends, as expected, on protein concentration. Indeed, higher protein concentrations enable higher oligomeric states but it seems that the cosolute type (potassium phosphate, Tris–acetate, Tris–HCl, and Mg^{2+}) has a stronger influence in determining the percentages of monomers, dimers, and octamers in solution. A single oligomeric species of TvEno Δ 34 could not be isolated under our experimental conditions, even by attempts using gel-filtration chromatography. The mixtures of species obtained do not show enzymatic activity, probably due to slight conformational changes involving both secondary and tertiary structures upon changing the buffer solution. In contrast, all combinations of oligomeric species show the ability to bind human plasminogen. According to 3D models of all three oligomeric species (monomer, dimer, and octamer), enolase should be prone to bind plasminogen by exposing the amino acid residues of the widely accepted putative plasminogen-binding motif (²⁶⁴FYDEEKLY²⁷²) to the surface (Figures 3 and S3). It has been described that the enolase–plasminogen complex is formed by non-native conformations of the partners.^{55,56} Conformations achieved in potassium phosphate, Tris–HCl, and Tris–acetate buffers might lose their nativeness to have enzymatic activity but are perturbed enough to be capable of binding plasminogen. The affinity toward plasminogen is variable and depends on the conformation of mixtures, but in all cases, either the hydrophobic environment of tryptophans or the secondary structure, or both are modified upon interaction. Additionally, the thermal stability of the combinations is variable. Remarkably, the unfolded state attained in the presence of phosphate ions maintains an important amount of secondary structure since the CD signal of the profiles under these conditions does not vary substantially. As expected, Mg^{2+} stabilizes TvEno Δ 34, probably by enabling higher-order oligomeric states.

T. vaginalis is a very primitive parasite with a large genetic multiplicity that^{57,58} provides the parasite the ability to respond to drastic environmental changes (such as temperature, microflora, pH, iron concentration, polyamines, zinc, host immune responses, and other unknown factors) by modulating the expression of multiple genes. In *T. vaginalis*, there are nine genes encoding enolase. Here, we found that the product of the gene TVAG_329460, which was described as a plasminogen receptor, is capable of forming several oligomeric states in equilibrium under nonharsh conditions. This is not the first time that glycolytic enzymes from *T. vaginalis* have

shown distinctive self-association characteristics compared with their orthologs from other biological species. An interesting example corresponds to triose phosphate isomerase (TvTIM). In *T. vaginalis*, there are two isoforms of this enzyme (TvTIM1 and TvTIM2), which differ by four amino acids in their protein sequences. They share enzymatic parameters and overall scaffolds, but they display substantial differences in their stabilities. TvTIM1 is more stable and less dissociable than TvTIM2. The differences in sequence and mainly in stability provide alternative functions to a TIM barrel protein as a laminin- and fibronectin-binding protein.^{59–61} This is one of the simplest examples of functional differentiation upon mutation.

CONCLUDING REMARKS

Monomers, dimers, and octamers of enolase from *T. vaginalis* coexist in solution in a variety of mild experimental conditions. As expected, the oligomeric state depends strongly on the protein concentration but it is not the main variable, dictating the self-association of enolase from *T. vaginalis*. The cosolute (buffer-salt and cofactor) importantly determines the dynamic self-association equilibria of TvEno.

Nevertheless, important questions must be addressed. How does a cosolute participate in the formation of different oligomeric states? Do different oligomeric states coexist within the cell? Which is the series of events taking place to adopt different oligomeric states? Are the mixtures capable of performing alternative functions of this protein? Is the oligomeric state of TvEno a type of function-regulation mechanism? Does the quaternary structure define the moonlighting function of enolase? All of these questions should be addressed with further investigation.

MATERIALS AND METHODS

Purification of Enolase. The gene identified as TVAG_329460 (UniProt entry A2EBB4) was amplified from genomic DNA of *T. vaginalis*. The polymerase chain reaction (PCR) primers were 5'-aac ggg aat cat atg aag gct ctc atc gag aag gtt g-3' (forward) and 5'-gag gac gga tcctta ttc ctc agc gag cat gtc-3' (reverse). Using these primers, the amplified sequence corresponds to an N-terminal-truncated version of enolase, eliminating 34 amino acid residues; therefore, the protein construct is depicted as TvEno Δ 34 (Figure S3). The PCR product was cloned into the *NdeI* and *BamHI* restriction sites (underlined) of a modified pET19 vector (courtesy of Professor Tom Ellenberger, Washington University School of Medicine). The plasmid pET19:TVAG_329460 Δ 34 was used to transform the Rosetta II strain of *Escherichia coli*. Cultures were grown to an optical density of 0.6 (600 nm) in 2xYT medium (plus 100 μ g L⁻¹ ampicillin and 34 μ g L⁻¹ chloramphenicol) at 37 °C. TvEno Δ 34 protein expression and purification were performed as described earlier.¹⁹ Protein homogeneity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%). After purification, enolase was extensively dialyzed against each buffer used: Tris-HCl, Tris-acetate, or potassium phosphate at 50 mM and pH 8.0 and stored at 4 °C for no more than 2 weeks.

Enzymatic Activity. The coupled reactions of rabbit muscle pyruvate kinase (PYK) and beef heart lactate dehydrogenase (LDH) were used to test enolase activity by following the decrease of NADH absorbance at 340 nm using a Scinco S-3100 UV-vis spectrophotometer. Standard activity

assays were performed at 25 °C in a 1.0 mL reaction mixture containing 100 mM triethanolamine HCl, pH 7.4; 1.9 mM 2-PGA; 1.3 mM ADP; 0.12 mM β -NADH; 2 mM MgSO₄, and 100 mM KCl. The auxiliary enzymes PYK and LDH were used at final activities of 7 and 10 U mL⁻¹, respectively, and were purchased from Sigma. Recombinant enolase TvEno Δ 34 was added at a final concentration of 6.5 μ g mL⁻¹. One activity unit is defined as the conversion of 1 μ mol of substrate per min. The Michaelis constant (K_M) of enolase for 2-PGA was determined using the abovementioned reaction conditions by varying the concentration of 2-PGA between 1.9 nM and 1.9 mM.⁸ Kinetic parameters were calculated from Michaelis-Menten plots by curve-fitting of experimentally determined data using the Origin program. Positive control experiments were performed using 0.025–0.05 units of commercial enolase from *S. cerevisiae* (ScEno), purchased from Sigma, without any further purification.

Homology Modeling of Different Oligomeric States of Recombinant Enolase from *T. vaginalis*. The 3D models of enolase from *T. vaginalis* of monomer, dimer, and octameric conformations were obtained using the SWISS-MODEL server (<https://swissmodel.expasy.org/interactive>).²⁰ Modeling templates were searched by using BLAST. The atomic coordinates of *S. pneumoniae* enolase were retrieved from the Protein Data Bank (PDB ID: 1W6T²¹). PyMOL program (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) was used to identify plasminogen-binding sites and tryptophan residues of each conformation.

CD Spectroscopy and Thermal Denaturation Transitions Were Monitored. CD measurements were performed in a JASCO J-815 spectropolarimeter (Jasco Inc., Easton, MD) equipped with a PFD-425S Peltier-type cell holder for temperature control and magnetic stirring. CD spectra were recorded from 200 to 250 nm using 1.0 cm path length cells. Ellipticities are reported as the mean residue ellipticity. Thermal denaturation transitions were followed by continuous monitoring of changes in ellipticity at a fixed wavelength of 220 nm, whereas the temperature of the sample was increased at a constant heating rate (2 °C min⁻¹). Actual temperatures within the cell were registered with the external cell holder probe. Cooling profiles were recorded after denaturation transitions had been completed. The temperature of heating and cooling profiles was registered and controlled through the Peltier accessory.

Fluorescence. Fluorescence spectra were obtained using an LS-55 Spectrofluorometer (PerkinElmer) equipped with a water-jacketed cell holder for controlling the temperature at 25 °C. The path length of quartz cuvettes was 1.0 cm. Spectra were recorded using two excitation wavelengths, 280 and 295 nm (as stated), and the emission spectra were collected from 320 to 400 nm and from 335 to 450 nm, respectively. The spectral center of mass (SCM) was calculated using the following equation

$$\text{SCM} = \frac{\sum (\lambda \times I_\lambda)}{\sum I_\lambda} \quad (1)$$

where I_λ represents the intensity data at each wavelength (λ) in the interval recorded.²²

Small-Angle X-ray Scattering (SAXS). Small-angle X-ray scattering (SAXS) experiments were carried out by means of a Kratky camera coupled to a copper anode X-ray tube whose $K\alpha$ radiation was selected with a nickel filter. The samples were introduced into a capillary tube. The SAXS intensity data, $I(q)$,

were collected with a linear proportional counter at a distance of 25 cm from the sample. In each case, the corresponding background was subtracted. Afterward, the data were processed with ITP program.^{23–27} The momentum transfer or scattering vector is defined as $h = (4\pi \sin \theta)/\lambda$, where 2θ is the scattering angle and λ is the beam wavelength. The shapes of the particles were determined by the shape of the Kratky curve,²⁵ and the size distributions of the particles were calculated.

Interaction with Plasminogen. The evaluation of plasminogen binding was tested by differential far-UV-CD and intrinsic fluorescence spectroscopies. Plasminogen human plasma was purchased from Sigma and used without any further purification at a molar concentration equivalent to the enolase concentration in terms of a monomer. The buffers used and experimental parameters were the same as stated above.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02197.

Representative Kratky plots and (B) size distribution function of enolase diluted in Tris–HCl buffer at a concentration of 300 $\mu\text{g mL}^{-1}$ (Figure S1); intrinsic fluorescence-emission spectra (excitation wavelength 280 and 295 nm) of native recombinant enolase TvEno Δ 34 (Figure S2); sequence of TvEno Δ 34 from *T. vaginalis*, predicted secondary structures are indicated (Figure S3); far-UV circular dichroism and intrinsic fluorescence-emission (excitation wavelength 295 nm) spectra of native human plasminogen, demonstrating different secondary and tertiary structure (Figure S4) (PDF)

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Notes

The authors declare no competing financial interest. Authors attest that this article is original and they agree with the publication of the manuscript without any ethical issues involved.

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