Dissociation of the Octameric Enolase from
* S. Pyogenes* - One Interface Stabilizes Another

Farhad Karbassi1*, Veronica Quiros1, Vijay Pancholi2, Mary J. Kornblatt1*

1 Department of Chemistry and Biochemistry, Concordia University, Montreal, Quebec, Canada, 2 Department of Pathology, Ohio State University, Columbus, Ohio, United States of America

Abstract

Most enolases are homodimers. There are a few that are octamers, with the eight subunits arranged as a tetramer of dimers. These dimers have the same basic fold and same subunit interactions as are found in the dimeric enolases. The dissociation of the octameric enolase from *S. pyogenes* was examined, using NaClO4, a weak chaotrope, to perturb the quaternary structure. Dissociation was monitored by sedimentation velocity. NaClO4 dissociated the octamer into inactive monomers. There was no indication that dissociation of the octamer into monomers proceeded via formation of significant amounts of dimer or any other intermediate species. Two mutations at the dimer-dimer interface, F137L and E363G, were introduced in order to destabilize the octameric structure. The double mutant was more easily dissociated than was the wild type. Dissociation could also be produced by other salts, including tetramethylammonium chloride (TMACI) or by increasing pH. In all cases, no significant amounts of dimers or other intermediates were formed. Weakening one interface in this protein weakened the other interface as well. Although enolases from most organisms are dimers, the dimeric form of the *S. pyogenes* enzyme appears to be unstable.

Introduction

Enolase (EC 4.2.1.11) catalyzes the reversible dehydration of 2-phosphoglycerate (PGA) to phosphoenolpyruvate (PEP), an essential reaction in both glycolysis and gluconeogenesis. Depending on the species, enolases have other functions, ranging from being a transcription factor in several organisms [1–3] to being a plasminogen receptor on the surface of many cells [4]. Both the primary sequence and the tertiary structure of this protein are strongly conserved. Enolase from most organisms exists as a dimer, with subunit molecular masses of 40–50 kDa. Crystal structures are now available for dimeric enolases; formation of the dimer involves the same regions of the two monomers as in the dimeric enolase (Fig. 1A). The dimer-dimer contacts (Fig. 1C) are mainly between identical portions of the two subunits; a region of the small domain contacts the same region in the other subunit, while the end of one helix in the large domain contacts the same region in the other subunit. The N-terminal of each subunit is located towards the center of the ring, while the C-terminal domains form the outer ring.

The active site is completely contained within the monomeric structure, leading to the question: are enolase monomers active? The answer appears to depend on the source of the enolase and the conditions used for dissociation. Dissociation of yeast enolase by chaotropic salts produces monomers that are inactive [13,14], but have normal secondary structure. Dissociation by dilution and increased temperature produces active monomers [15,16]. Dissociation by imidazole or low pH produces monomers with low activity [17]. This variability may indicate that the structure necessary for activity is stabilized by subunit interactions and easily lost in their absence.

We are interested in determining the effects of quaternary structure on the function of enolase; we chose to study enolase from *S. pyogenes*. Although a crystal structure is not available for this enolase, it shares 97% homology with that of *S. pneumoniae*, and has been shown, both by mass spectrometry and by modeling, to be octameric [18]. Our goals were to dissociate the octameric enzyme into monomers and dimers in order to compare the octameric, dimeric, and monomeric forms of this enolase. To our surprise, we were not able to produce dimeric enolase.

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* E-mail: judithk@alcor.concordia.ca

* Current address: Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada

Abstract

Dissociation of the Octameric Enolase from *S. Pyogenes* - One Interface Stabilizes Another.
Results

Dissociation and Inactivation by NaClO₄

We have previously shown that NaClO₄ dissociates yeast enolase into monomers [19]. We therefore incubated the wild type octameric enolase from *S. pyogenes* in varying concentrations of NaClO₄ and used analytical ultracentrifugation (AUC), in the sedimentation velocity mode, to examine the quaternary structure of the protein (Fig. 2A). In buffer, 84–90% of the protein sedimented with an s₂₀,ₐ of 14.4. Smaller species (1% or less) and larger species (10–15%), including aggregates, were also present. In 0.2 M NaClO₄, 2 species, with s₂₀,ₐ values of 14.0 and 3.48, were present in almost equal amounts (49% and 41% respectively). As the NaClO₄ concentration was increased still further, the large peak decreased and the small increased. The major species present in the absence of NaClO₄ has an s₂₀,ₐ of 14.48 ± 0.09 (average of three determinations) and a hydrodynamic radius, by DLS, of 6.42 ± 0.04 nm (average of three determinations). The molecular mass calculated from these values is 452 ± 7 kDa, which corresponds to a nonamer. The same AUC data, analyzed by DCDT+ and fitting for s₂₀,ₐ and mass, yields mass values that correspond to septamers. Since it has been sown by mass spectrometry that the enolase from *S. pneumoniae* is an octamer [18], we conclude that the major species present in the absence of NaClO₄, is an octamer. We conclude that the species with s₂₀,ₐ of 3–4 is the monomer, since we have previously shown that the monomer of yeast enolase has an s₂₀,ₐ of 3.35 [19]. The s₂₀,ₐ values for these two species are relatively constant over the range of NaClO₄ used in these experiments, but they do decrease slightly, which may indicate that changes in tertiary structure are also occurring. Only 4% of the protein was present as intermediate sized species, with s₂₀,ₐ values in the range of 6–9, as can be seen in Fig. 2B. These may correspond to dimers (the dimer of yeast enolase has an s₂₀,ₐ of 5.5 [19]) and/or larger species. The fact that these are present in small amounts and that, in the AUC profiles, there is not complete separation of these species means that there is considerable variation in both the s₂₀,ₐ values and the relative amounts of the various species. By varying the NaClO₄ from 0 to 0.4 M, we obtained a complete picture of the dissociation process. Fig. 3 shows the concentrations of octamer, monomer and intermediates as a function of the NaClO₄ concentration.

![Figure 1. The structure of the octameric enolase of *S. pneumoniae* (1W6T.pdb). A) The dimeric unit showing the monomer-monomer interface, viewed down the β-barrel of the large domain of one subunit. The green ball is the Mg²⁺ at the active site. B) The octamer, with one dimer colored as in Fig. 1A. C) Close up of the dimer-dimer interface, showing F137 (bottom of the figure) and E362 (top pair of residues); residue 362 in *S. pneumoniae* = residue 363 in *S. pyogenes*. One dimer is blue and the other is green, as in Fig. 1B. All figures were made with Chimera (www.cgl.ucsf.edu/chimera/). doi:10.1371/journal.pone.0008810.g001](https://www.plosone.org/doi/10.1371/journal.pone.0008810.g001)

![Figure 2. Sedimentation velocity data for the dissociation of the octameric enolase by NaClO₄. The concentrations of the various species in the sample are shown as a function of their sedimentation coefficients. Prior to centrifugation, the samples were incubated for 18 hours at 15 °C in TME (solid line), TME containing 0.2 M NaClO₄ (long dashes) or TME containing 0.28 M NaClO₄ (dash-dot-dot). All samples contained 2.7 μM enolase. Centrifugation and data analysis were performed as described in Methods. Fig. 2B is an enlargement of the data shown in Fig. 2A. doi:10.1371/journal.pone.0008810.g002](https://www.plosone.org/doi/10.1371/journal.pone.0008810.g002)
Incubation in NaClO4 also inactivated the enzyme. This loss of activity correlated closely with formation of monomers (Fig. 4), suggesting that all of the various species, with the exception of monomers, were active.

Effects of Mutations at the Dimer-Dimer Interface

Since one of our original objectives was to study the dimeric form of the S. pyogenes enolase, we studied a variant that had two mutations, F137L and E363G, at the dimer-dimer interface (Fig. 1C). Our assumption was that these mutations would weaken the dimer-dimer interface, but have no effect on the monomer-monomer interface. This variant was expressed, and purified. Based on the circular dichroism (CD) spectrum in the peptide bond region, the double mutant was correctly folded; the specific activity of the double mutant in our standard enzymatic assay was 90–95% that of the wild type enzyme. The effects of NaClO4 on the activity and quaternary structure of this variant were then studied. The changes at these two positions destabilized the structure of the protein. 50% dissociation and inactivation occurred at about 0.06 M NaClO4, as opposed to 0.2 M for the wild type protein (Fig. 4). As with the wild type enzyme, dissociation of the octamer into monomers was accompanied by formation of only small amounts of intermediate species (Fig. 5). The Tm for denaturation for this variant, monitored by CD at 220 nm, was decreased, from 68°C for the wild type to 61°C.

Both dissociation and inactivation of the variant form were largely, but not completely, reversible. Enolase, at 19.4 μM, after incubation in 0.2 M NaClO4, had 32% of its original activity and was 21% octameric. Following a 24-hour dialysis against buffer, the protein was 77% octameric and had 74% of its original activity.

Questions and Other Experimental Approaches

Are these results due to the perchlorate ion acting as an oxidizing agent? The fact that both dissociation and inactivation are largely reversible means that neither process is due to irreversible covalent modifications of the protein, such as oxidation. Are these results due to the presence of Na+, a known inhibitor of enolase [20]? In order to answer this question, the ability of other salts to dissociate the octameric enolase was examined. These experiments were all performed using the F137L/E363G variant. The effects of TMACl were qualitatively the same as those of NaClO4; the octameric enolase was dissociated into monomers (Fig. 6); the maximum concentration of intermediates was 3%. Although not studied in detail, NaCl and KCl had the same effects as TMACl. In 0.4 M NaCl or KCl the enzyme was 70–80% monomeric. All three salts also inactivated the enzyme. However, 0.4 M NaOAc neither dissociated nor inactivated the enzyme. These results establish that dissociation of the octameric enolase into monomers is not a specific effect of either ClO4− or Na+. Dissociation is produced by salts of

Figure 3. Distribution of species versus concentration of NaClO4. Data were obtained as described in Fig. 2. Data for each concentration of NaClO4 came from separate incubations and AUC runs, performed over a period of several days. All samples contained 2.7 μM enolase. Solid circles, % octameric; open circles, % monomeric; solid triangles, % all intermediate species.
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Figure 4. Dissociation and inactivation of the wild type octameric enolase and the F137L/E363G variant by NaClO4. Enolase, 2.7 μM was incubated in NaClO4 as described in Figs. 2 and 3. Prior to loading the AUC cells, each sample was assayed for enzymatic activity. Open symbols, % activity; closed symbols, % non-monomeric protein (calculated at 100% - % monomeric); circles, F137L/E363G variant; triangles, wild type enolase.
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Figure 5. Distribution of species versus concentration of NaClO4 for the F137L/E363G variant. Data were obtained from experiments performed as described in Figs. 2 and 3. All samples contained 2.7 μM enolase. Solid circles, % octameric; open circles, % monomeric; solid triangles, % all intermediate species.
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chaotropic ions [21], such as ClO$_4^-$ and Cl$^-$, but not by salts of kosmotropic ions such as Oac$^-$. Dissociation also occurred if the pH was increased. At pH 7.4, the variant enzyme was 88% octameric; at pH 9.0, it was 39% octameric, with 2% intermediates and 54% monomers. By pH 10 in glycine buffer, it was 88% monomeric; less than 1% was present as intermediate species. As with salts, this dissociation was not accompanied by formation of intermediates. High pH also inactivated the enzyme.

Does dissociation involve denaturation? Dissociation, whether by NaClO$_4$ or by high pH, was not accompanied by loss of secondary structure, as determined by peptide bond circular dichroism (CD) (Fig. 7). (In contrast, complete dissociation of the wild type enzyme, which requires 0.4 M NaClO$_4$, was accompanied by partial unfolding.)

Time Course of Dissociation and Inactivation

The above experiments were all performed under equilibrium conditions. In order to examine more closely the correlation between dissociation and inactivation and to look for evidence of a step-wise process, we wished to follow the time course of both processes. In a dynamic light scattering (DLS) experiment, the intensity of the signal from large molecules is greater than that from small molecules. Dissociation of the octameric enzyme will result in a decrease in the intensity of the signal. NaClO$_4$ was added to a sample of enzyme and the DLS signal was monitored as a function of time. In a parallel experiment, NaClO$_4$ was added to enzyme, and aliquots were removed and assayed at various times. As can be seen in Fig. 8, the decrease in DLS signal and the loss of activity followed the same time course; in addition, there was no indication that multiple processes were occurring.

Discussion

Sedimentation velocity experiments clearly showed that incubation in NaClO$_4$ produced changes in the quaternary structure of S. pyogenes enolase; the octameric enzyme was dissociated into monomers that were inactive. We have shown that both the inactivation and dissociation are reversible. However, the fact that we observe discrete species in the AUC experiments indicates that equilibration among the species is slow.

The time course of dissociation, monitored by DLS, and the time course of inactivation are very similar. Both sets of data (inactivation vs. time and DLS intensity vs. time) in Fig. 8 can be fit to an equation for single exponential decay (fit not shown). There is no indication in this data of the occurrence of multiple steps or the presence of significant amounts of intermediates.

The octameric protein dissociates into monomers, without the formation of large amounts of dimers. In the various dissociation experiments that we performed, the maximum concentration of the intermediate species was less than 10% of the total protein and was observed when dissociation was almost complete (Fig. 3 and 5). This is not the behavior one would expect of a true intermediate and may indicate that these intermediates are formed from the association of monomers. The fact that there is no
intermediate whose concentration builds up and then decreases suggests either of two pathways: 1) the octamer dissociates directly into monomers or 2) the octamer dissociates into some smaller species which then rapidly dissociates into monomers. Both dissociation and inactivation have a dependence on protein concentration. With the variant enzyme, the concentration of NaClO₄ necessary for 50% dissociation is 0.06 M when the protein concentration is 2.7 μM but increases to 0.12 M when the protein concentration is 22.7 μM. However, this concentration dependence is not great enough to be due to an octamer dissociating into 4 dimers or 8 monomers, but could be explained by an octamer dissociating into two tetramers.

We have previously shown that yeast enolase is dissociated by NaClO₄; under the same concentrations used in these experiments, complete dissociation of the yeast enzyme requires 0.3 M NaClO₄. The monomer-monomer interface of the S. pneumoniae enolase is similar to that of other enolases in the amount of buried surface area; groups contributing hydrogen bonds or electrostatic interactions in the yeast dimer interface [5] are also present in the S. pyogenes and S. pneumoniae enolases. The two interfaces of the octameric enolase from S. pneumoniae are similar in amount of buried surface and the ratio of charged to hydrophobic residues (1.1 for the monomer-monomer interface and 0.8 for the dimer-dimer interface) [10]. Although NaClO₄ is a chaotropic salt, it will also weaken electrostatic interactions. Thus the fact that complete dissociation of the S. pyogenes enolase into monomers also occurred at 0.3 M NaClO₄ was not surprising.

What was surprising were the results with the F137L/E363G variant. We fully expected that this variant would be dissociated into dimers, since both changes are at the dimer-dimer interface (Fig. 1C). This variant was more easily dissociated than was the wild type, but did not dissociate into dimers. These changes have destabilized both the dimer-dimer and the monomer-monomer interface. The dimer-dimer interface stabilizes the monomer-monomer interface, in effect stabilizing the active, functional unit.

Are these results specific to NaClO₄? We have shown that dissociation can also be produced by incubation of the enzyme with NaCl, KCl, and TMACl, but not with NaOAc. Dissociation by salts was not an effect of Na⁺ or K⁺ ions, nor of high ionic strength; it did require a chaotropic salt. Dissociation could also be produced by alkaline pH. In all of these cases, the characteristics of the dissociation are the same – the octamer dissociated into monomers with little formation of dimers or other intermediates, the monomers were inactive, and there was no loss of secondary structure.

The vast majority of enolases are dimeric, with the same monomer-monomer interface that is seen in the octameric enolases. Yeast enolase is fully dimeric with K_diss = 1.5 × 10⁻⁶ M [19]. Enolases from a small number of organisms are octameric, with eight subunits arranged as a tetramer of dimers, forming a ring (Fig. 1A). There are other enzymes which, depending on source, occur in multiple quaternary states. Cytosolic creatine kinases are dimers, while the mitochondrial creatine kinase is an octamer [22] formed by 4 dimers [23]. Phosphoglycerate mutase from vertebrates is dimeric while the enzyme from yeast is tetrameric [24], and dihydrodipicolinate synthase can be either tetrameric or dimeric [25]. In all of these cases, the larger form of the enzyme can be dissociated into dimers, either by changing conditions or by introducing mutations. Arginine decarboxylase of E. coli exists as either a decamer or as a dimer, with the equilibrium between the two forms shifted by pH [26]. Formiminotransferase-cycloleaminase is also an octamer, with the subunits arranged in a ring as a tetramer of dimers [27]. This protein can be dissociated by urea into dimers; depending upon conditions the urea treatment, either subunit interface can be disrupted producing either of two types of dimers [28].

In these examples, the two types of interfaces present in the oligomer appear to be independent - one can be disrupted while maintaining the other. This is not the case for enolase from S. pyogenes. When we weakened one set of interactions by mutations we weakened the other set as well; there is cooperativity between the two interfaces.

With the octameric enolase, there are differences between the octamer and monomer in the aromatic CD spectrum, fluorescence and binding of 8-anilino-1-naphthalene sulfonic acid (data not shown). We have previously shown that dissociation of yeast enolase produced subtle changes in the structure of the monomer; there was a small increase in disordered structure and several peptide bonds, including one in a helix far from the interface, became susceptible to proteolysis [19]. However, none of these observations shed light on the nature of the communication between the two interfaces. The monomer-monomer interface in S. pyogenes enolase involves the small domain of one subunit and the large domain of the other. The dimer-dimer interaction mainly involves small domain with small domain. Communication between the two interfaces could be via the two faces of the small domain and/or via the orientation of the large domain relative to the small.

Enolases have not been obvious drug targets, since enolase is an essential enzyme and all enolases studied to date use the same mechanism. Recently, there have been suggestions that small structural differences between enolases could be exploited to selectively inhibit enolases from parasitic protozoa [6,29]. In addition to its essential role in glycolysis, enolases from many pathogenic bacteria are also plasminogen receptors [30–32]. To date, there is little information about the relationship between enolase structure and plasminogen binding, although one mutation that partially dissociates enolase appears to increase binding [18]. Compounds designed to disrupt the dimer-dimer interface of the S. pyogenes enolase should have no effect on the dimeric mammalian enolases. The potential of such compounds to affect infectivity or plasminogen binding are worth examining.

Methods

Mutagenesis, Expression and Purification

The gene for enolase from S. pyogenes had previously been cloned and inserted into the pET-14b plasmid [33]; the resulting plasmid codes for a protein with an N-terminal his-tag. E. coli strains XL1Blue and BL21(DE3) were used for storage and expression of the plasmid respectively. The protein coded for by the initial plasmid differed from the wild type protein at two positions: 137 and 363. These two positions were changed to the wild type positions by mutagenesis, using the QuickChange method (Stratagene, La Jolla, CA, USA) and oligonucleotides from Invitrogen (DDO, Quebec, Canada). Enzymes for molecular biology were from Fermentas Canada (Burlington, Ont. Canada). The presence of the desired mutations was verified by sequencing the gene (BioS&T, Inc., Lachine, Canada) and by Q-ToF mass spectrometry (CBAMS, Concordia University, Canada). Protein with the correct sequence is referred to as wild type enolase; protein expressed from the original plasmid is referred to as the F137L/E363G variant.

E. coli, BL21(DE3), containing the desired plasmid, was grown in Luria broth containing ampicillin (BioShop Canada, Inc, Burlington, Ont. Canada) at 37°C until an OD₆₅₀ of 0.6 was reached. Isopropylthiogalactopyranoside (BioShop Canada Inc.) was added to a final concentration of 0.5 mM, the temperature was lowered to 29°C and the cells grown for an additional 4 hours. The cells were...
harvested by centrifugation and stored at −20°C. The cells were thawed, suspended in washing buffer (20 mM HEPES, 300 mM NaCl, 10 mM imidazole, 1 mM Mg(OAc)₂, pH 7.5) containing 0.06 mg mL⁻¹ of DNase and 0.06 mg mL⁻¹ of RNAse and sonicated. The crude extract was centrifuged and loaded onto a Ni-NTA column (Qiagen, Inc., Mississauga, Ont., Canada). Following washing, the protein was eluted with elution buffer (20 mM HEPES, 300 mM NaCl, 250 mM imidazole, 1 mM Mg(OAc)₂, pH 7.5). The protein was immediately dialyzed overnight against a 100-fold excess of TME buffer (50 mM Tris/HCl, 1 mM Mg(OAc)₂, 0.1 mM protein was immediately dialyzed overnight against a 100-fold excess of TME buffer. All of the above steps were performed at 4°C.

Enzymatic Assays

The enzyme activity was monitored by following the conversion of PGA to PEP at 240 nm. The assay buffer contained 50 mM HEPES, pH 7.5, 1 mM Mg(OAc)₂, and 1 mM PGA; assays were performed at room temperature. Since Na⁺ inhibits other enolases [20], the HEPES was titrated with KOH. Activity measurements are the average of duplicates, except for the experiment in Fig. 5. The concentration of enolase was determined from the absorbance at 280 nm and expressed as concentration of subunits. Extinction coefficients were calculated from the amino acid composition [34]. For the wild type enzyme and the double mutant, ε = 43320 M⁻¹ cm⁻¹. PGA was synthesized enzymatically from ATP and glyceric acid [35].

Dissociation of Enolase

Enolase was incubated in TME buffer containing varying concentrations of NaClO₄ or other salts for 18–24 hours at 15°C; the activity of the enolase was measured and the samples were used for the various biophysical measurements. When pH was varied, 50 mM Tris or Glycine, containing 1 mM Mg(OAc)₂ and 0.1 mM EDTA, was adjusted to the desired pH at 20°C. Incubations of the enzyme in these buffers were also at 20°C. The concentration of enolase in most experiments was either 2.7 μM monomers or 22.7 μM monomers. For experiments monitoring the time course of changes, the measurements of activity and light scattering were begun within 1 minute of adding NaClO₄ to the sample.

Analytical Ultracentrifugation

Sedimentation velocity experiments were performed in a Beckman (Fullerton, CA, USA) XL-I analytical ultracentrifuge at the Concordia University Center for Structural and Functional Genomics. Samples were centrifuged at 35,000 rpm in a Ti60 rotor for 12–16 hours at 15°C, except for the pH studies which were performed at 20°C, and monitored at either 290 nm or 280 nm. Data were analyzed using SEDFIT [36] (available at www.analyticalultracentrifugation.com), using the continuous c(s) distribution model. To determine the mass of the undissociated species, a few AUC runs were also analyzed by DCDT+, version 2.02 (available from J. Philo at www.jpilo.mailway.com), fitting for s₂₀,w and mass. The partial specific volume of the protein and the viscosity and density for each buffer were calculated using SEDNTERP, version 1.07 (D.B. Hayes, T. Laue and J. Philo, available at www.bbri.org/RASMB/rasmb.html) and used to convert sedimentation coefficients to s₂₀,w.

Circular Dichroism and Dynamic Light Scattering

Circular dichroism spectra were recorded on a Jasco J-815 spectropolarimeter, with a thermostatically controlled sample compartment. The concentration of these protein was 4.1 μM. Samples, at 20°C, were scanned from 260 to 200 nm at 20 nm per second with a 1 nm bandwidth and a 1 second response time. Five spectra were recorded and averaged. Spectra of the corresponding buffers were recorded using the same parameters. The Jasco software was used for subtraction of the buffer spectra and smoothing. For temperature denaturation studies, the samples were monitored at 220 nm over a temperature range of 35°C to 80°C at a rate of 15°C per hour.

DLS measurements were made on a Dyna Pro instrument (Wyatt Technology, Santa Barbara, CA, USA), with the cell compartment thermostated at 15°C. Protein concentration was 2.8 μM. 10-second acquisitions, with a laser power of 100%, were recorded continuously for 6 hrs.

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Author Contributions

Conceived and designed the experiments: MJK. Performed the experiments: FK VQ MJK. Analyzed the data: FK MJK. Contributed reagents/materials/analysis tools: VP. Wrote the paper: MJK. Supervised the work of E. Karbassi and V. Quiros: MJK.

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