May 18, 2006

Stabilization of α -chymotrypsin at air–water interface through surface binding to gold nanoparticle scaffolds

Brian J. Jordan

Rui Hong

Basar Gider

Jason Hill

Todd S. Emrick, *University of Massachusetts - Amherst*, et al.

Stabilization of α -chymotrypsin at air–water interface through surface binding to gold nanoparticle scaffolds†

Brian J. Jordan,^a Rui Hong,^a Basar Gider,^a Jason Hill,^a Todd Emrick^b and Vincent M. Rotello^{*a}

Received 17th March 2006, Accepted 13th April 2006

First published as an Advance Article on the web 18th May 2006

DOI: 10.1039/b603980d

Gold nanoparticles stabilize chymotrypsin (ChT) against denaturation at the air–water interface through catenation and preferential localization of the nanoparticles at the air–water interface with concomitant decrease in interfacial energy.

Enzymes are highly specific and efficient biocatalysts with a wide range of biotechnological and industrial applications.¹ The amphiphilic nature of proteins, however, leads to their instability at air–water and oil–water interfaces.² Dynamic process conditions that expose proteins to these interfacial environments such as transport, printing, and mixing can cause irreversible protein denaturation with concomitant loss of activity.³ One way of increasing the stability of proteins is through immobilization of enzymes onto solid supports.⁴ Monolayer-protected nanoparticles (MPNs) offer an excellent alternative to conventional immobilization techniques. The surface properties of MPNs can be readily tailored through structural changes in the monolayer.⁵ Moreover, these MPNs feature extremely high surface to volume ratios, making them excellent candidates for biocatalytic applications.⁶

In previous studies, we have demonstrated the electrostatic binding of α -chymotrypsin (ChT) to anionic nanoparticle surfaces featuring tetra(ethylene glycol) carboxylate ligands (AuTCOOH, Fig. 1).⁷ Significantly, the native structure of ChT is retained upon complexation with AuTCOOH. More importantly, the enzyme–nanoparticle complex is still active towards neutral and cationic substrates,⁸ facilitating the application of these enzyme–nanoparticle conjugates as biocatalysts. We report here the stabilization of ChT at air–water interfaces through non-covalent binding to AuTCOOH nanoparticles. Two mechanisms have been identified for this stabilization: (1) restriction of ChT unfolding through multivalent electrostatic interactions and (2) preferential localization of AuTCOOH at the air–water interface.

Reproducible exposure of ChT to the air–water interface was performed by vigorous stirring under controlled conditions (electronic supplementary information, ESI†). ChT denaturation occurred due to air bubbles that were introduced into the vortex of the solution; the shear effects associated with stirring were determined to have a negligible effect on ChT activity as little

Fig. 1 Schematic representation of ChT and ChT–AuTCOOH complex at air–water interface.

change in enzyme efficiency was observed in stirred degassed solution (see ESI†).

In our initial studies, we used circular dichroism (CD) as a structural probe for the stability of ChT and the ChT–AuTCOOH (Fig. 2a). These studies were performed using a 4 : 1 ratio of ChT to AuTCOOH, providing an excess of nanoparticle based on gel binding assays that indicated an 11 : 1 stoichiometry for the ChT–AuTCOOH complex (ESI†). Prior to stirring, both ChT and the ChT–AuTCOOH complex displayed similar CD features with minima at 202 nm and 232 nm, corresponding to the native secondary and tertiary structure of the protein.

Upon stirring ChT rapidly, ChT is completely denatured, as indicated by the disappearance of the minimum in the CD spectrum at 232 nm and a blue shift of the minimum at 202 nm to 198 nm. However, in the presence of AuTCOOH, the ChT structure was clearly retained even after 90 min of stirring. The minimum at 232 nm diminished with time, but the overall structure of ChT was maintained as demonstrated by the minimal shift of the minimum at 202 nm. Taken together, these studies indicate that AuTCOOH stabilizes the enzyme structure upon exposure to the air–water interface.

The fraction of *completely* folded ChT in solution was quantified by the retention of absorbance intensity of the Trp(141) residue at 232 nm.⁹ Utilizing a two state model, (native $\rightleftharpoons$ denatured), the percentage of folded protein is

^aDepartment of Chemistry, University of Massachusetts, 710 N. Pleasant Street, Amherst, MA, 01003, USA.

E-mail: rotello@chem.umass.edu; Fax: +1-413-545-4490

^bDepartment of Polymer Science and Engineering, University of Massachusetts, Amherst, MA

† Electronic supplementary information (ESI) available: experimental protocols, gel electrophoresis of ChT–AuTCOOH complex, activity of ChT and ChT–AuTCOOH at elevated ionic strength solutions. See DOI: 10.1039/b603980d

proportional to the ellipticity at 232 nm in the CD spectrum. Using this method, the denaturation rate constant for ChT alone is $0.9 \times 10^{-3} \text{ s}^{-1}$, three-fold greater than that of ChT complexed with AuTCOOH ($0.3 \times 10^{-3} \text{ s}^{-1}$, Fig. 2b).

Fig. 2 (a) CD spectra of ChT (3.2 μM) and ChT–AuTCOOH (4 : 1 ratio) after vigorously stirring. (b) Rate of ChT unfolding, assuming a two state model, native or denatured. F_t corresponds to the percentage of native protein, calculated from the absorbance at 232 nm in the CD spectra.

The stabilization of ChT structure at the air–water interface by AuTCOOH was concomitant with the conservation of enzymatic activity. ChT activity was evaluated by monitoring the catalytic hydrolysis reaction of neutral substrate *N*-benzoyl-tyrosine-*p*-nitroaniline (BTNA). ChT alone lost its activity over a period of about 20 min under continuous stirring conditions. Again, assuming a two-state model, the rate constant for the loss of activity of ChT was $4.9 \times 10^{-3} \text{ s}^{-1}$ with a half-life of 2.4 min (Fig. 3). This rate is somewhat faster than the rate of secondary structure change, presumably because of the sensitivity of active site geometry to structural changes. Upon the addition of AuTCOOH, the rate of ChT activity decay was much slower. The rate constant for ChT activity at a 1 : 1 (ChT : AuTCOOH) ratio was determined to be $1.4 \times 10^{-3} \text{ s}^{-1}$, a nearly 4-fold decrease in the rate of activity loss. Taken together, these studies indicate a substantial increase in the stability of ChT when bound to AuTCOOH, thus demonstrating that the AuTCOOH nanoparticles stabilize ChT at the air–water interface. Salt effect studies further support this conclusion; stabilization was not observed when ChT and AuTCOOH nanoparticles were stirred in solutions of elevated ionic strength (ESI), in accord with prior studies that show that high ionic strength disrupts ChT–nanoparticle binding in ionic solutions.^{7, 8}

Two potential mechanisms for protein stabilization by AuTCOOH can be readily identified: electrostatic stabilization through catenation¹⁰ and decrease of interfacial energy through

Fig. 3 Rates of ChT (0.8 μM) activity decay of ChT alone and ChT with varying concentrations of AuTCOOH.

selective localization of the nanoparticles at the interface. Protein stabilization *via* catenation has been observed after adsorption to silicate materials through electrostatic interactions between the protein and surface.¹¹ This mode of stabilization is consistent with the results observed at the 11 : 1 (ChT : AuTCOOH) stoichiometry, where we expect the ChT to be completely complexed based on our gel studies.

Significantly, further stabilization of ChT was observed at higher AuTCOOH : ChT ratios. This trend suggests a second stabilization mechanism that is dependant on AuTCOOH concentrations. Colloidal nanoparticles have been shown to stabilize liquid–liquid interfaces, such as toluene–water interface by reducing the interfacial energy between the two immiscible liquids.¹² AuTCOOH may also localize to and hence stabilize the air–water interface,¹³ thereby reducing the exposure of ChT at the air–water interface and protein denaturation.

To investigate the affinity of ChT and AuTCOOH for the air–water interface, changes in surface tension upon addition of ChT and AuTCOOH were measured (Du Nouy ring method). In these studies, lower surface tension signifies a greater affinity for the air–water interface with concomitant lowering of interfacial energy.¹⁴ ChT (10 μM) decreased the surface tension of phosphate buffered solution (5 mM, pH = 7.4) by $\sim 5\%$, from $69.4 \pm 1.0 \text{ dynes cm}^{-1}$ to $65.7 \pm 0.7 \text{ dynes cm}^{-1}$. As expected, ChT exhibits some affinity for the air–water interface. Addition of AuTCOOH (10 μM) resulted in a larger ($\sim 20\%$) decrease in the surface tension of the buffered solution from $69.4 \pm 1.0 \text{ dynes cm}^{-1}$ to $56.4 \pm 1.3 \text{ dynes cm}^{-1}$, demonstrating that AuTCOOH nanoparticles exhibit a higher degree of stabilization of the air–water interface than ChT. Importantly, the ChT–AuTCOOH complex (1 : 1, 10 μM) exhibited an almost identical surface tension ($58.8 \pm 1.2 \text{ dynes cm}^{-1}$) as AuTCOOH, strongly suggesting the preferential localization of the particle surface at the interface. As there was no stabilization observed without ChT–AuTCOOH binding, this preference most likely arises from asymmetric disposition of the ChT on the particle surface.¹⁵

In summary, we have demonstrated that monolayer protected gold nanoparticles AuTCOOH stabilize ChT at the air–water interface *via* multivalent surface binding. AuTCOOH exhibits a stronger segregation to the air–water interface relative to ChT,

further protecting ChT from interfacial denaturation. This study thus presents a viable strategy to stabilize enzymes under conditions found in real world biocatalytic applications.

Acknowledgements

This research is supported by the National Institute of Health (Grant GM 52259, V. M. R.) and the National Science Foundation (CHE-0239486, T. E.). B.J.J. graciously acknowledges an NSF IGERT fellowship (DUE-044852).

Notes and references

- 1 A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts and B. Witholt, *Nature*, 2001, **409**, 258; F. Bordusa, *Chem. Rev.*, 2002, **102**, 4817; E. Katz and I. Willner, *Angew. Chem., Int. Ed.*, 2004, **43**, 6042.
- 2 G. Y. Zhu and P. Wang, *J. Am. Chem. Soc.*, 2004, **126**, 11132; P. A. Gunning, A. R. Mackie, A. P. Gunning, N. C. Woodward, P. J. Wilde and V. J. Morris, *Biomacromolecules*, 2004, **5**, 984.
- 3 G. M. Nishioka, A. A. Markey and C. K. Holloway, *J. Am. Chem. Soc.*, 2004, **126**, 16320.
- 4 U. T. Bornscheuer, *Angew. Chem., Int. Ed.*, 2003, **42**, 3336; T. Arakawa, S. J. Prestrelski, W. C. Kenney and J. F. Carpenter, *Adv. Drug Delivery Rev.*, 2001, **46**, 307.
- 5 N. W. Fadnavis, V. Bhaskar, M. L. Kantam and B. M. Choudary, *Biotechnol. Prog.*, 2003, **19**, 346; U. Drechsler, N. O. Fischer, B. L. Frankamp and V. M. Rotello, *Adv. Mater.*, 2004, **16**, 271; C. V. Kumar and A. Chaudhari, *Chem. Mater.*, 2001, **13**, 238.
- 6 J. C. Love, L. A. Estroff, J. K. Kriebel, R. G. Nuzzo and G. M. Whitesides, *Chem. Rev.*, 2005, **105**, 1103; P. Pengo, Q. B. Broxterman, B. Kaptein, L. Pasquato and P. Scrimin, *Langmuir*, 2003, **19**, 2521; M. G. Ryadnov, B. Ceyhan, C. M. Niemeyer and D. N. Woolfson, *J. Am. Chem. Soc.*, 2003, **125**, 9388.
- 7 R. Hong, N. O. Fischer, A. Verma, C. M. Goodman, T. Emrick and V. M. Rotello, *J. Am. Chem. Soc.*, 2004, **126**, 739.
- 8 R. Hong, T. Emrick and V. M. Rotello, *J. Am. Chem. Soc.*, 2004, **126**, 13572.
- 9 C. R. Cantor and S. N. Timasheff, *The Proteins*, Academic Press, Inc., New York, 1982.
- 10 A. P. Golovanov, G. M. Hautbergue, S. A. Wilson and L. Y. Lian, *J. Am. Chem. Soc.*, 2004, **126**, 8933; B. J. Palla and D. O. Shah, *J. Colloid Interface Sci.*, 2002, **256**, 143.
- 11 M. Hartmann, *Chem. Mater.*, 2005, **17**, 4577; S. Robinson and P. A. Williams, *Langmuir*, 2002, **18**, 8743; R. Ravindra, S. Zhao, H. Gies and R. Winter, *J. Am. Chem. Soc.*, 2004, **126**, 12224.
- 12 Y. Lin, H. Skaff, T. Emrick, A. D. Dinsmore and T. P. Russell, *Science*, 2003, **299**, 226; F. Reincke, S. G. Hickey, W. K. Kegel and D. Vanmaekelbergh, *Angew. Chem., Int. Ed.*, 2004, **43**, 458; H. W. Duan, D. A. Wang, D. G. Kurth and H. Mohwald, *Angew. Chem., Int. Ed.*, 2004, **43**, 5639.
- 13 E. Glogowski, J. B. He, T. P. Russell and T. Emrick, *Chem. Commun.*, 2005, 4050.
- 14 K. Norgaard, M. J. Weygand, K. Kjaer, M. Brust and T. Bjornholm, *Faraday Discuss.*, 2004, **125**, 221; N. Spredi, P. Di Profio, L. Marte, S. Bufali, L. Brinchi and G. Savelli, *Eur. J. Biochem.*, 2001, **268**, 6491; T. Y. Lin and S. N. Timasheff, *Protein Sci.*, 1996, **5**, 372.
- 15 C. J. Beverung, C. J. Radke and H. W. Blanch, *Biophys. Chem.*, 1999, **81**, 59; P. Viparelli, F. Alfani, A. Gallifuoco and M. Cantarella, *J. Mol. Catal. B: Enzym.*, 2004, **28**, 101.