Reversible Regulation of Chymotrypsin Activity Using Negatively Charged Gold Nanoparticles Featuring Malonic Acid Termini

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Abstract: Negatively charged gold nanoparticles featuring 2-(10-mercapto-decyl)-malonic acid were synthesized using the Murray place-displacement reaction. These water-soluble malonate gold mixed monolayer protected clusters (MMPCs) effectively bind and inhibit chymotrypsin based on complementary electrostatic surface recognition. The effect of increasing ionic strength on inhibition was also studied. It was observed that addition of high ionic strength solutions to protein-nanoparticle complexes show almost complete restoration of protein activity. The conformational change of chymotrypsin upon binding to the MMPC was investigated using fluorescence spectrometry and circular dichroism, thus correlating structural changes with enzyme activity.

INTRODUCTION

Protein surface recognition is an important area of study for the regulation of protein-protein interactions and protein-nucleic acid interactions involved in cellular processes [1]. The regulation of proteins with well defined active sites have been well studied through inhibition with small molecule systems [2]. The binding of the protein surface, however, provides a more difficult target due to the large surface area and multiple types of interactions which make the task a challenging one [3]. The binding of protein surfaces provide a new approach for the regulation of proteins that lack well defined active sites [4].

An area of recent research has been the use of mixed monolayer protected clusters (MMPCs) for biomacromolecular surface recognition [5]. MMPCs are readily synthesized using the Brust-Schiffrin reduction [6] followed by subsequent functionalization with the Murray place-displacement reaction [7]. The core sizes of these MMPCs can be easily tuned during the initial reduction step to sizes varying from 1.5 to 5.2 nm diameters [8]. These core sizes afford suitably scaled scaffolds comparable in size to biomacromolecular structures. The MMPC scaffold also provides useful functionality with the ability to functionalize the monolayer with multiple recognition groups, which have the ability for templation [9].

In our previous work, we have observed that MMPCs functionalized with mercaptoundecanoic acid (MUA) effectively inhibit chymotrypsin (ChT) [5b]. The inhibition of protein activity occurred through a two stage process; initially the binding occurs through a fast complementary electrostatic process. This is followed by a slow denaturation of the protein, presumably upon the hydrophobic alkyl core of the MMPCs monolayer causing a further decrease in the activity of the protein over time. To probe the initial electrostatic nature of the interaction, the protein was incubated in solutions containing various ionic strengths before the addition of the nanoparticle to the protein [11]. At high concentrations of NaCl (1.5 M), total disruption of the binding between the MMPC and the protein was observed. Further studies were performed where the protein-MMPC complex was incubated for 12 hours then various concentrations of NaCl were added. At high concentrations of NaCl (600 mM), the protein-MMPC complex was disrupted and maximum restoration of protein activity was observed. This activity was much lower than the native ChT in similar NaCl concentrations (~35%) presumably due to the denaturation of the protein over the incubation period.

To further study the interactions of various functional groups decorating MMPCs on the regulation of chymotrypsin, we have synthesized a nanoparticle functionalized with 2-(10-mercapto-decyl)-malonic acid (AuDA) through the Murray place-displacement reaction. The effect of the nanoparticle on the protein activity was studied through chromogenic assays. The structural affect of the nanoparticle binding to the chymotrypsin was studied through circular dichroism (CD) and fluorescence spectroscopy. The effect of ionic strength during the initial binding and on the disruption of the protein-nanoparticle complex was also studied.

AuDA

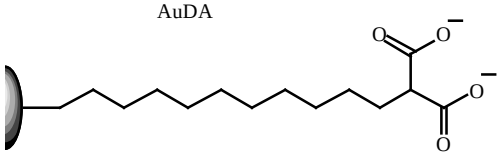

Fig. (1). Structure of AuDA.

EXPERIMENTAL

General

ChT (type II from bovine pancreas), β-galactosidase (β -gal) (grade VI from *Escherichia coli*) , *o*-nitrophenyl

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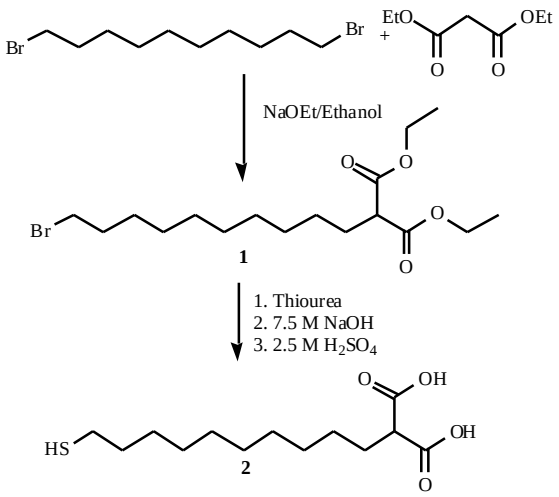
α-D-galactopyranoside (ONPG), and succinyl-Ala-Phe-*p*-nitroanilide (SPNA) were purchased from Sigma. Hydrogen tetrachloroauric acid was obtained from Strem Chemicals, Newton, MA. All other chemicals were purchased from Aldrich and used as received. ^{1}H NMR spectra for the ligand synthesis were obtained in $CDCl_3$ (purchased from Cambridge Isotopes) at 400 MHz and referenced internally to $CHCl_3$ at 7.28. ^{1}H NMR for the nanoparticle was obtained in D_2O (purchased from Cambridge Isotopes) at 400 MHz and referenced internally to HOD at 4.80. IR spectra were recorded as a thin drop-cast film on salt plates from $CHCl_3$. Mass spectral data were obtained at the University of Massachusetts Mass Spectrometry Facility which is supported, in part, by the National Science Foundation.

Synthesis of 2-(10-mercapto-decyl)-malonic Acid

2-(10-Bromo-decyl)-malonic acid diethyl ester (1)

Malonic diethyl ester (6.24 mmol) and sodium ethoxide (30% w/w, 6.24 mmol) were added to a solution of ethanol. 1,10-dibromodecane (31.2 mmol) was then added and solution stirred overnight. The solution was washed with water (3 times, 20 ml aliquots) and the organic layer was collected. The organic layer was dried over $MgSO_4$ and the solution was filtered. The filtrate was collected and reduced *in vacuo*. The resulting crude product was purified using silica gel column chromatography with a gradient starting with pure hexane to pure ethyl acetate (yield: 98.4 %). ^{1}H NMR δ: 4.21 (q, 4H), 3.43 (t, 2H), 3.32 (t, 1H), 1.88 (m, 4H), 1.28 (m, 20H).



Scheme (1). Synthesis of 2-(10-mercapto-decyl)-malonic acid.

2-(10-mercapto-decyl)-malonic acid (2)

1 (13.2 mmol) was added to a solution of ethanol containing thiourea (26.4 mmol). The solution was refluxed for 16 hours with stirring. The solvent was reduced *in vacuo* and a 7.5 M solution of aqueous NaOH (25 ml) was added. Argon was bubbled through the solution for 15 min and the solution was then stirred for 6 hours under argon at 60°C. The solution was then acidified with 2.5 M H_2SO_4 to pH 2 and extracted with ethyl acetate (3 times, 50 ml aliquots). The organic layers were combined and dried with $MgSO_4$, filtered and the filtrate was collected. The filtrate was reduced *in vacuo* to provide **2** as a white solid(yield: 89%). ^{1}H NMR δ: 3.46 (t, 1H), 2.54 (q, 2H), 1.96 (q, 2H), 1.62 (p, 2H), 1.35 (m, 14H) IR: 3300-2800, 3019, 2929, 2856, 1715 cm^{-1}. HRMS-FAB $[M+H]^+$: *m/z* 277.1468; calc'd for $[M+H]^+$ 277.1474.

Nanoparticle Synthesis (AuDA)

The malonic acid functionalized nanoparticle (AuDA) was synthesized by modifying our previously published procedure [11]: 2-(10-mercapto-decyl)-malonic acid was added to pentanethiol-functionalized nanoparticles (50 mg) in 6 ml of tetrahydrofuran. Argon was bubbled through the solution for 10 min, and the reaction was stirred for 2 days under argon. The solvent was then removed *in vacuo* and washed three times with 50 ml of dichloromethane, and the precipitate was collected by centrifugation. The AuDA was further solubilized in water upon treatment of the nanoparticle with small amounts of a NaOH (1M) solution in water until complete dissolution of the nanoparticle occurred. The water was then removed *in vacuo*. The functional group loading of the AuDA was determined by NMR endgroup analysis and found to be 100%.

Inhibition Assays

ChT (3.2 μM) was preincubated with varying concentrations of nanoparticles (50 nM-800 nM) in 5 mM sodium phosphate buffer (pH 7.4). Activity assay was modified from a previously reported procedure [12]. At established time points, 16 μl of succinyl-Ala-Phe-*p*-nitroanilide stock solution (25.9 mM) was added to 184 μl of above solutions, resulting in a final reaction concentration of 2 mM. Activity was followed by measuring the formation of *p*-nitroaniline every 15 s for 20 min at 405 nm with an Ultra Microplate Reader (EL808 Bio-Tek Instruments, Inc.). ChT stock solution (10 μM) was prepared from a lyophilized powder. AuDA stock concentration was 40 μM [13]. The total reaction volume was 200 μl for each well. Samples were studied in triplicate. Assays for β-galactosidase [14] were modified from previously described procedures and the AuDA was found to have no inhibitory effect.

Circular Dichroism (CD)

CD was performed on a Jasco 700 spectrophotometer, using a quartz cuvette with a 1-mm path length. ChT (3.2 μM) was incubated with AuDA (800 nM) in 5 mM sodium phosphate buffer (pH 7.4). CD experiments of ChT (3.2 μM) in 5 mM sodium phosphate buffer (pH 7.4) were also performed. Scans were taken from 190 to 250 nm at a rate of 5 nm/min, with a 0.1-nm step resolution and a 4-s response. Three scans were averaged at a constant temperature of 20°C, with a 5-min equilibration before the scans. Samples were left at room temperature overnight before reaching the 24-h time point. Data obtained with high tension voltage (HT) over 600 V were not included in the analysis. Thermally denatured ChT was obtained by heating a solution of ChT (3.2 μM) at 90 °C for 25 min.

Fluorescence

Fluorescence was performed on a Shimadzu RF-5301 PC spectrofluorophotometer using a quartz cuvette with a 1-cm

path length. Samples were excited at 295 nm, and the emission spectra were recorded from 300 to 450 nm. ChT (3.2 μM) was normalized by adding a non-interacting positively charged nanoparticle (800 nM) in 5 mM sodium phosphate buffer (pH 7.4) to account for the absorbance by the nanoparticle gold core. Fluorescence measurements of ChT (3.2 μM) in 5 mM sodium phosphate buffer (pH 7.4) with no nanoparticle were also performed. ChT (3.2 μM) was incubated with AuDA (800 nM) in 5 mM sodium phosphate buffer (pH 7.4). Fluorescence measurements were performed at 0 hr, 6 hr, and 24 hrs for all samples. No shift in the wavelength of the emission peak of ChT was observed with or without the positively charged nanoparticle present.

Ionic Strength Studies

ChT (3.2 μM) was incubated with AuDA (800 nM) in varying NaCl concentrations (10 mM-1 M) in 5 mM sodium phosphate buffer (pH 7.4) for 1 hour. 16 μl of succinyl-Phe-Ala-*p*-nitroanilide of a 25.9 mM dissolved in DMSO/ethanol (10%/90% v/v) was added to 184 μl of each sample, resulting in a final concentration of 2 mM. For the disassociation studies, ChT (3.2 μM) was preincubated with AuDA (800 nM) for 12 hours in 5 mM sodium phosphate buffer (pH 7.4). NaCl (40μl) was then added from different stock concentrations to afford final concentrations ranging from 10 mM to 1 M concentrations. Solutions were then incubated for one hour and activity was followed by measuring the formation of *p*-nitroaniline every 15 s for 20 min at 405 nm with an Ultra Microplate Reader (EL808 Bio-Tek Instruments, Inc.). ChT stock solution (10 μM) was prepared from a lyophilized powder. AuDA stock concentration was 40 μM [13]. The total reaction volume was 200 μl for each well. Samples were studied in triplicate.

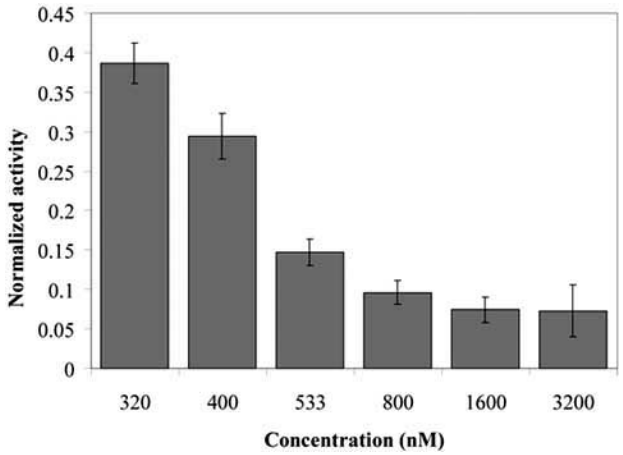


Fig. (2). Normalized activity of ChT bound to AuDA in various concentrations 1 hr after addition of the nanoparticle. Normalized activity is based on native ChT activity plotted *vs*. AuDA concentration. Error bars are from changes in activity over a 6 hour period.

RESULTS AND DISCUSSION

Inhibition Assays

To determine the ratio of AuDA to ChT necessary to achieve optimal inhibition of ChT activity various concentrations of AuDA were added to a fixed concentration of ChT. The samples were incubated for 1 hour and the activity of the ChT was observed by the hydrolysis of SPNA, a chromogenic substrate. At a ratio of 1:10 (AuDA:ChT) ~60% inhibition of the ChT was observed. At a ratio of 1:8 (AuDA:ChT) the inhibition increased to ~70% and at a concentration of 1:6 the inhibition increased further to ~85%. At a 1:4 ratio (AuDA:ChT), the inhibition of the protein achieved maximum levels as no further increase in inhibition was observed at higher ratios of 1:2 and 1:1 within error. It was also observed that the inhibition of the ChT did not increase over a six hour period suggesting a time independent inhibition.

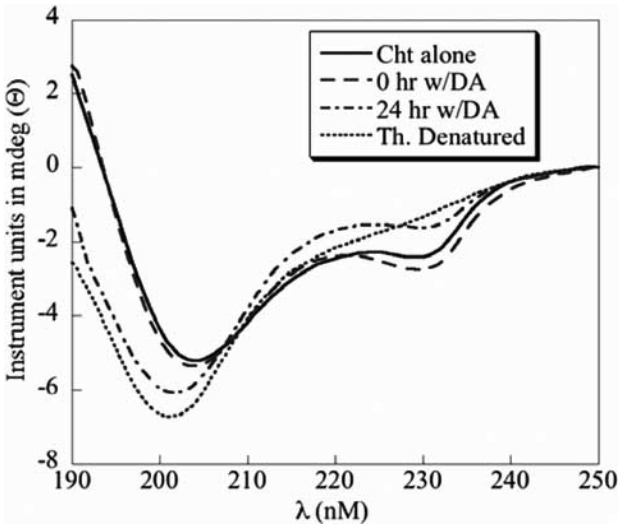


Fig. (3). Circular dichroism of native ChT, thermally denatured ChT, and time dependent measurements of ChT bound to AuDA. ChT was kept a concentration of 3.2 μM and AuDA was kept at a concentration of 800 nM. Spectra were smoothed for clarity.

Structural Studies with CD and Fluorescence

CD experiments were performed to observe any structural changes that may occur with ChT bound to AuDA. ChT was added to AuDA and the change in the CD spectrum of the bound ChT was observed over time. CD is a powerful probe to follow changes in protein structure, particularly for ChT which has a characteristic spectrum [15]. ChT has a distinguishing minimum around 230 nm due to an aromatic perturbation (apparently due to Trp 141), [16] which has been correlated with activity [17]. In thermally denatured ChT this minimum disappears and activity of the protein is completely diminished [17]. Upon addition of AuDA there is actually a slight increase in the minima at 230 nm which has been associated with surfactant or salt stabilization of the protein to a more active form [10]. However, as reported above the AuDA is observed to inhibit ChT activity ~90%. Over a period of 24 hours incubation of the chymotrypsin with the nanoparticle, there is a reduction of the minima at 230 nm, suggesting a conformational shift towards the thermally denatured form of the ChT. Further, a blue shift is observed in the minimum at 208 nm suggesting a disordering of the ChT to a more random coil, similar to the thermally denatured ChT.

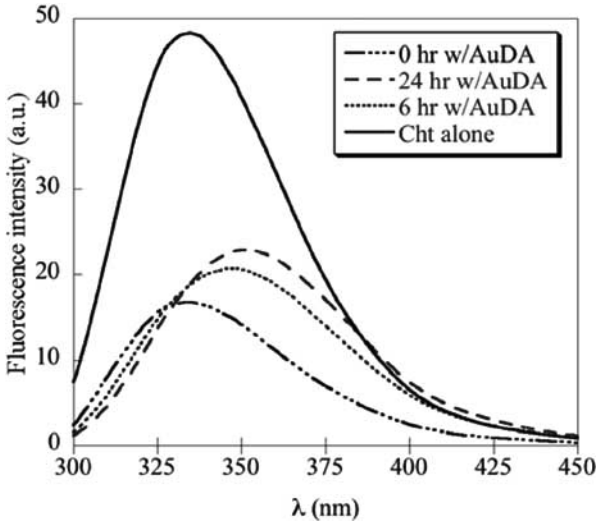


Fig. (4). Fluorescence spectra of ChT normalized with a non-interacting nanoparticle and spectra of ChT bound to AuDA over a period of time. ChT concentration was 3.2 μM and AuDA concentration was 800 nM.

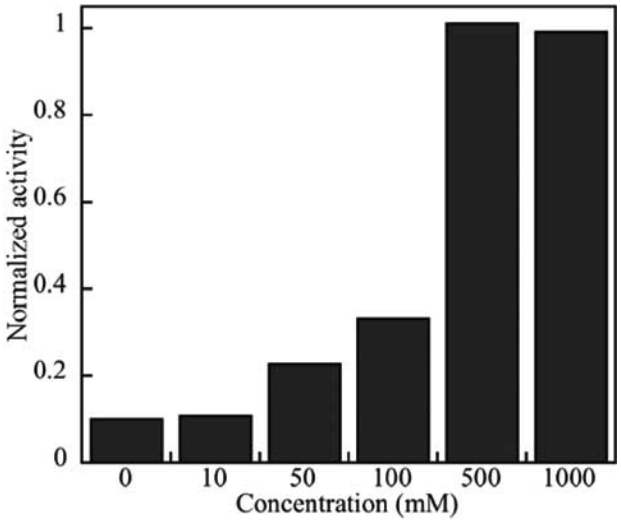


Fig. (5). Effect of various ionic strengths on the initial binding of AuDA to ChT. Normalized activity is based on activity of ChT at similar concentrations of NaCl plotted *vs*. NaCl concentration. ChT concentration was 3.2 μM and AuDA concentration was 800 nM.

To further probe the structural changes of ChT bound to AuDA, fluorescence measurements were performed. It is well known that tryptophan emission wavelength is dependent on the nature of the local environment [18]. In more polar environments the emission band of tryptophan is red shifted relative to emission in a hydrophobic environment. In ChT there are eight tryptophan residues with six on the surface of the protein and two buried in the hydrophobic core [19]. This provides a powerful probe for following structural changes in ChT where one can infer unfolding of the ChT upon a red shift in the tryptophan emission band.

Due to the absorbance of the gold core during fluorescence measurements, the native ChT has been normalized with a non-interacting gold nanoparticle to account for this absorbance. Upon addition of the nanoparticle to the ChT, the fluorescence intensity immediately decreases due to further quenching of the fluorescence by the nanoparticle core suggesting that the protein is now bound to AuDA with no immediate shift of the emission band observed. After a six hour incubation of the ChT with AuDA, a considerable red shift of the emission band is observed to around 348 nm and upon further incubation to 24 hours, the emission band shifts further to ~352 nm, similar to thermally denatured chymotrypsin. This suggests that the protein has unfolded, exposing the tryptophan residues in the core to the more polar aqueous environment. This corresponds to the CD data where there was an observed shift in the spectrum to more disordered or random coil confirmation.

Effect of Ionic Strength Upon Binding

To probe the effect of salt concentrations on the binding process, various solutions of ChT with NaCl concentrations ranging from 10 mM to 1 M were prepared. AuDA was then added to the solutions and the activity of the protein was observed after a 1 hr incubation period during which time optimum inhibition of ChT (~90%) is observed with no NaCl present. The activity of the protein was observed by the hydrolysis of SPNA, a chromogenic substrate mentioned earlier in the inhibition studies. The activity was normalized to ChT at similar NaCl concentrations and was plotted against NaCl concentration Fig. (**5**). It has been observed that certain concentrations of NaCl, other salts, and surfactant stabilize chymotrypsin and enhance its activity [20]. A similar trend was observed in this study with higher concentrations of NaCl enhancing activity of ChT by up to 100% at 1M NaCl. This enhanced activity has been accounted for with control experiments at similar concentrations of NaCl and ChT alone to allow for normalization of the data.

As observed in our experiments initial lower concentrations of NaCl have little effect on the inhibition of the ChT. Observable changes in inhibition begin to occur between 50 mM and 100 mM NaCl where ~35% of the native ChT is obtained. At 500 mM NaCl, the binding of AuDA to ChT is completely disrupted with full retention of ChT activity. This provides further evidence supporting the hypothesis that the initial binding is based on electrostatic complementarity.

In addition to disrupting ChT-AuDA complexation, the increase of salt concentrations to preincubated ChT-AuDA complexes should disrupt the complex. To study the effect of increasing ionic strength on the nanoparticle-protein complex, the nanoparticle was incubated with the protein for 12 hr. NaCl solutions were added to individual samples to afford final concentrations ranging from 10 mM to 1M NaCl. These samples were then incubated for one hour and the activity of the ChT was observed. Upon addition of NaCl there was considerable disruption of the ChT-AuDA complex. The greatest increase in activity of the protein occurred between 100 mM to 200 mM. Upon further increase of the NaCl concentration, a steady increase in

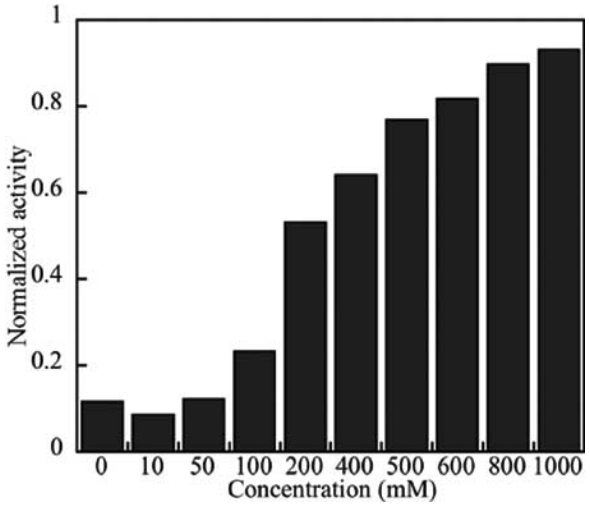


Fig. (6). Data obtained after 12 hr incubation period and 1 hour after addition of salt. Data were normalized to ChT activity vs. NaCl concentration.

activity of the ChT occurs until almost complete disruption of the binding is observed upon addition of a final concentration of 1 M with recovered activity of ~90%. This reactivation suggests that the binding is largely electrostatic and the large recovery of activity is perhaps due to the incomplete denaturation of the protein as observed in the CD spectrum especially at the 230 nm minimum.

In summary, we have shown that nanoparticle functionalized with 2-(10-mercapto-decyl)-malonic acid is an effective agent for surface recognition of ChT. It was observed that it is an efficient inhibitor of ChT at biologically relevant concentrations. There is an observable unfolding and disordering of the protein as observed by CD and fluorescence measurements. Increasing ionic strength has also been observed to have a great effect on the initial binding of the nanoparticle to ChT. Also, addition of the NaCl to preincubated solutions of ChT with AuDA observe an almost complete restoration of activity at around 800 mM of NaCl.

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