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New Strategy for Reversible Modulation of Protein Activity through Site-Specific Conjugation of Small Molecule and Polymer

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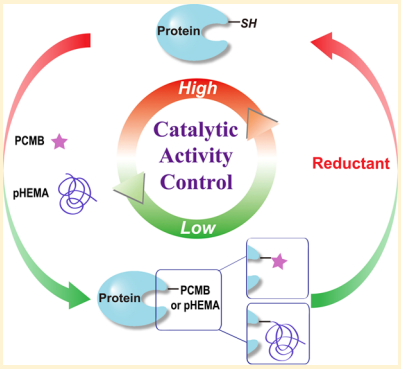
Supporting Information

ABSTRACT: A new strategy for accurate and reversible modulation of protein activity via simple conjugation of the sulfhydryl modifier and polymer with the introduced Cys residue in protein was developed in this study. With *Escherichia coli* inorganic pyrophosphatase (PPase) as a model protein, we used site-directed mutagenesis to generate a mutant PPase (PPC) with a substituted Cys residue at the specific Lys-148 site, which is within a conserved sequence near the active site and exposed to the surface of the PPC for chemical reaction. The site-specific conjugation of the mutated Cys residue in PPC with sulfhydryl modifier *p*-chloromercuribenzoate (PCMB) and pyridyl disulfide-functionalized poly(2-hydroxyethyl methacrylate) (pHEMA) resulted in obvious decrease or complete loss of the catalytic activity of PPC, due to the conformational change of PPC. Compared with the effect of small molecule modification (PCMB), the pHEMA conjugation led to greater inhibitory effect on protein activity due to the significant change of the tertiary structure of PPC after conjugation. Moreover, the protein activity can be restored to different extents by the treatment with different amount of reductive reagents, which can result in the dissociation between PPC and PCMB or pHEMA to recover the protein conformation. This study provides a new strategy for efficient control of protein activity at different levels by site-specific conjugation of a small molecule and polymer.



INTRODUCTION

Protein activity control is the critical issue for human health and disease treatment. For example, the activity levels of adipogenesis-related Wnt protein,[1] breast cancer-related BRCA2 protein,[2] and diabetes-related TORC2 protein[3] play a dominate role in the formation of these diseases. The ability to accurately and reversibly control protein activity at different levels could provide new opportunities for disease therapy, molecular diagnostics, and tissue engineering. The development of simple and efficient tools for accurate control of protein activity is one of the greatest challenges for these applications and therefore it has received much attention recently. Under in vivo conditions, the regulation of protein activity is achieved either by conformational changes caused by the binding of different ligands or by covalent modifications through feedback mechanisms.[4−7] However, the in vivo strategies for controlling protein activity are difficult to use in vitro. For in vitro studies, protein activity is generally regulated by altering the environmental conditions of the protein solution, such as temperature, pH value, and ionic strength;[8−11] however, the alteration of these conditions often affects protein stability and causes irreversible inactivation of the protein.[12,13]

Several studies have discovered that some proteins change their activity in response to the redox state of the environment,[14] and the sulfhydryl group of the Cys residue in the protein is directly involved in a series of redox reactions.[15−17] For example, the sulfhydryl group can be oxidized to form disulfide bonds and then reduced back to sulfhydryl groups under redox conditions and each of these states corresponds to a different protein conformation, leading to the modulation of protein activity.[18] Nevertheless, the change in protein activity based on the redox state is associated with the number of Cys residues in the protein and their location within the structure.[19−21] Polymer conjugation, as another method for regulating protein activity in vitro, has received great attention in recent years. Many studies have been conducted to regulate protein activity by conjugation of stimuli-responsive polymers.[22] For example, Hoffman et al. reported that conjugated *N,N*-dimethylacrylamide (DMA)/*N*-4-phenylazo-phenylacrylamide (AZAAm) copolymers with streptavidin and endoglucanase 12A modulated its activity by changes in temperature or light conditions;[23,24] Sumerlin et al. demonstrated that the BSA activity can be controlled by conjugation of temperature-responsive polymer PNIPAm, in which the conformation change of polymer chains between the expanded state and collapsed state caused by temperature can control protein activity,[25] but in these cases the control of protein activity was kept in a narrow range. Researches revealed that the regulation of protein activity by polymer conjugation is closely related to the location of the polymer chain on the protein, and site-

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specific conjugation of polymer with protein is beneficial for accurate control of protein activity.[22,26,27] Therefore, in this study, we expected to explore the potential of site-specific conjugation with small molecule and polymer for effective regulation of protein activity.

PPase is an important protein that provides energy and phosphate substrates for biosynthesis reactions by catalyzing the hydrolysis of pyrophosphate.[28] There are only two Cys residues in the *E. coli* PPase, and they are buried within the three-dimensional protein structure and show no significant response to changes in the redox state of the environment.[29] Therefore, we used site-directed mutagenesis to introduce an additional Cys residue into the specific Lys148 site, which is within a conserved sequence near the active site and is exposed to the surface of the PPase for chemical reaction. The site-specific conjugation of the mutated Cys residue in PPC with sulfhydryl modifier *p*-chloromercuribenzoate (PCMB) and pyridyl disulfide-functionalized poly(2-hydroxyethyl methacrylate) (pHEMA) resulted in obvious decrease or complete loss of the catalytic activity of PPC. Circular dichroism (CD) measurement determined that the effect of PCMB and pHEMA on PPase activity was caused by a conformational change of protein. Compared with the effect of small molecule modification (PCMB), the pHEMA conjugation led to greater inhibitory effect on protein activity due to the significant change of the tertiary structure of PPC after conjugation. Moreover, the protein activity can be restored to different extents by treatment with different amounts of reductive reagents, which can result in the dissociation between PPC and PCMB or pHEMA to recover the protein conformation.

RESULTS AND DISCUSSION

Chemical Properties of Wild Type and Variant Protein. There are two Cys residues in *E. coli* PPase; these residues are buried within the three-dimensional structure of the protein molecule and show very low reactivity in the natural state. These residues are inaccessible to chemical reagents.[29] To use the Cys residue to reversibly regulate protein activity, an additional Cys residue with little or no influence on protein activity was introduced directly on the surface of the PPase. Lys148 is located in a flexible loop which forms the wall of the PPase active site; this amino acid is a conserved sequence out of the active center in Family I PPases.[30] Site-directed mutagenesis indicated that the K148R mutation did not affect protein activity.[31] Therefore, the Lys residue was chosen for mutation to a Cys residue. First, the *ppa* gene from the *E. coli* K-12 strain was amplified by PCR and cloned into the expression vector pQE30. The AAG bases of the Lys148 codon were substituted with TGC, and a mutant *ppa* gene was obtained. DNA sequencing confirmed the existence of the mutation (Supporting Information, Figure S1). After protein expression was induced with isopropyl *β*-D-1-thiogalactopyranoside (IPTG), a 21 kDa protein band appeared on an SDS-PAGE; this band was consistent with the desired protein size and indicated the expression of the wild type PPase (PP) and the PPC variant. The expression products were purified by Ni-affinity chromatography and ultrafiltration. Purified PP and PPC were obtained (Figure 1).

PPase catalyzes the hydrolysis of inorganic pyrophosphate (PPi) into inorganic phosphate (Pi). Its catalytic mechanism primarily relies on the lysine, arginine, tyrosine, aspartic acid, and glutamic acid residues in the active center. The Cys residue does not participate in the catalytic reaction. Because only one

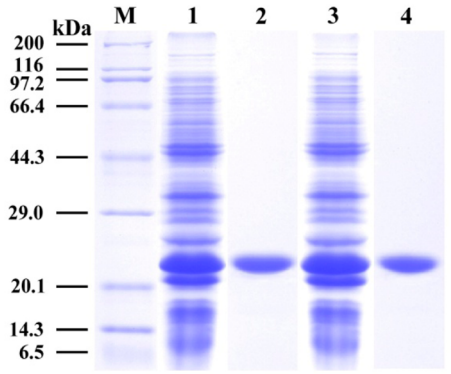

Figure 1. SDS-PAGE analysis of the expressed PPase. Lane M, standard protein marker. Lanes 1 and 3, total cell protein from induced *E. coli* cultures expressing PP and PPC. Lanes 2 and 4, purified PP and PPC. The proteins were electrophoresed on a 4−12% gradient gel and stained with Coomassie blue.

amino acid site on the random structure of the protein surface was changed (Lys148 replaced with a Cys residue), the secondary structure of the protein was not affected except for a slight shift of the tertiary structures (Supporting Information, Figure S2). Moreover, the biological activity of the protein also remained similar to that of the WT-PPase. The PPase catalytic activity assay for the hydrolysis of sodium pyrophosphate demonstrated that the specific activity of PP was 8.57 kat/kg, and the activity of PPC was 9.56 kat/kg, which was slightly improved compared to that of PP (Table 1). We also found

Table 1. Chemical Properties of the PP and PPC

protein	pI	MW	free thiol content	specific activity (kat/kg)
PP	5.73	20970.8	4.8 ± 2.0%	8.57 ± 0.19
PPC	5.61	20945.8	104.4 ± 5.2%	9.56 ± 0.31

that the ion selectivities of these two PPases were similar. Mg^{2+} was the optimal ion for the maximum catalytic activity, whereas other metal ions, such as Mn^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, Ni^{2+}, and Cu^{2+}, inhibited the PPase activity (Supporting Information, Table S1). Therefore, it can be concluded that the mutation had little impact on protein activity and that the catalytic mechanism of the variant was nearly the same as that of the wild type. As expected, with the introduction of the Cys residue on the surface of the molecule, the reactive free thiol content of PPC increased to 104%, but that of PP was only 5% (Table 1). As a result, the sulfhydryl group of the newly introduced Cys residue on the variant surface can be used to regulate protein activity.

Conjugation of PCMB and pHEMA to PPC. PCMB is a commonly used specific sulfhydryl modifier. It can effectively react with the sulfur atom of the sulfhydryl group via its mercury atom, leading to the formation of a mercury−sulfur linkage, which causes the direct and specific modification of Cys residues within protein molecules.[32,33] PCMB modification of the mutant Cys148 in PPC was performed (Scheme 1). Based on Ellman's assay on the PPC−PCMB conjugate, we confirmed that 91.4% sulfhydryl groups were reacted with PCMB for modification. With the addition of cysteine, the reductive reagent, to the PPC−PCMB solution, the reactive free thiol content on PPC was recovered to the extent of 83.3%. These results indicate that PCMB was conjugated to PPC via chemical linkage of the mercury−sulfur bond, and the removal of the

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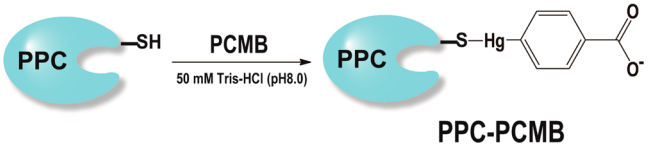
Scheme 1. Conjugation of PCMB with PPC



specific PCMB modification from the protein by cysteine treatment, resulting from the cleavage of mercury−sulfur bond between PCMB and PPC, and leads to the return of the original structure of the protein.

Pyridyl disulfide group can be used for preparing protein−polymer conjugates via the specific interaction of the pyridyl disulfide group at the end of the polymer chain and sulfhydryl group in protein surface.[34,35] Tao et al. synthesized poly(*N*-(2-hydroxypropyl) methacrylamide) (PHPMA) with a pyridyl disulfide midchain for protein−polymer conjugation.[36] Herein, an ATRP initiator with a pyridine disulfide end group was synthesized according to the literature procedure,[35] and then used for ATRP of HEMA to introduce the functional group at the end of the pHEMA. Compared with other synthesized water-soluble polymers for protein conjugation,[24,37] the advantage of pHEMA is that it is water-insoluble; therefore, the unreacted polymers after protein conjugation can be precipitated by dialysis against water and then easily removed by centrifugation, which significantly simplified the separation process. The as-prepared pyridyl disulfide-functionalized pHEMA (P-pHEMA) was verified by ^{1}H NMR and GPC (Supporting Information, Figure S3), and the information on the synthesized P-pHEMA was described in Table 2.

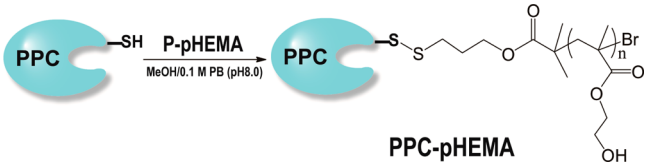
Table 2. Characteristics of the Pyridyl Disulfide Polymers[a]

[HEMA]/[Initiator]	time (min)	conversion (%)	Mn (theory)	Mn (^{1}H NMR)	PDI (GPC)	% end group content (^{1}H NMR)
50/1	90	89.4	6160	8019	1.24	89

[a]Polymerization conditions: [HEMA] = 50 v/v %, CH_3OH, 23 °C, [Initiator]:[CuBr]:[Bpy] = 1:1:2.

The P-pHEMA was conjugated to PPC via the coupling of pyridine disulfide end group with sulfhydryl group in PPC (Scheme 2). The formation of PPC-pHEMA conjugates was

Scheme 2. Conjugation of P-pHEMA with PPC



verified by SDS-PAGE (Figure 2A). The gel clearly showed a shift of the conjugates (lane 2) compared to PPC (lane 1). The formation of disulfide linkage between the PPC and pHEMA was confirmed by dithiothreitol (DTT) treatment. The shift in peak from the PPC-pHEMA band to the PPC band was distinctly observed (lane 4). This result demonstrates that the polymer was conjugated with protein through reducible disulfide bonds. The dimensional change during the conjugation was monitored by DLS measurement. As shown in Figure 2B, pHEMA conjugated PPC (PPC-pHEMA) showed an increase in hydrodynamic diameter compared to the nonconjugated one (PPC) due to the conjugation, and recovered to PPC's original diameter with DTT treatment. The shift of hydrodynamic diameter also supported the fact that the pHEMA was site-specifically conjugated to the sulfhydryl group in PPC by reversible disulfide bonds.

Influence of PCMB and pHEMA Conjugation on Protein Activity. The influence of different kinds of conjugation molecules on protein activity was investigated. Figure 3 shows the influence of PCMB and pHEMA conjugation on the catalysis activity of PPC. We observed an obvious decrease of the PPi hydrolysis activity of PPC by PCMB treatment, and this trend increased with the reaction time. For example, the enzymatic activity decreased to 21% after PCMB treatment for 2 h and stabilized with extended conjugation times, indicating that modification of the sulfhydryl group by PCMB was saturated. In contrast, the enzymatic activity of the PP did not significantly change with PCMB treatment. These results indicate that the Cys residue at the 148 site of the PP introduced by site-directed mutagenesis caused the PPC to become sensitive to the sulfhydryl modifier, and PCMB treatment led to a large decrease in the enzymatic activity as a result of the specific modification of the Cys sulfhydryl group by PCMB.

However, pHEMA conjugation had a much greater effect on the PPi hydrolysis activity of PPC. The enzymatic activity of PPC was almost lost (0.37% of nonconjugated PPC) after pHEMA conjugation in 0.5 h. The activity of PPC treated with methanol and PBS buffer mixture for 30 min showed no significant difference from that of PPC without treatment (98.12 ± 1.61%), which means that methanol has no obvious influence on protein activity in such a short reaction time. These results demonstrate that the introduction of polymer led to the change of protein activity from "on" to "off", showing a greater inhibitory effect on protein activity compared with the conjugation of small molecules. Additionally, the activity of PPC conjugated by pHEMA with a larger molecular weight ($M_{n,HNMR}$ = 25049) is 0.33% compared with that of the nonconjugated PPC, which is similar to the activity of PCC conjugated by pHEMA with a smaller molecular weight ($M_{n,HNMR}$ = 8019) (Supporting Information Table S2).

Activity Recovery of PPC−PCMB and PPC−pHEMA Conjugates by Reductive Reagents. The modification of sulfhydryl groups on proteins by PCMB is reversible, and the modified protein can be returned to its original structure by the treatment of a reductive reagent, such as cysteine.[38] Similarly, the disulfide linkage between the pHEMA and protein can also be cleaved by DTT treatment to restore protein's structure.

With addition of the reductive reagent cysteine to PPC−PCMB, the protein activity was recovered to varying degrees (Figure 4). In addition, the recovery efficiency was dependent on the concentration of cysteine. When the cysteine concentration was 3 mM in the reaction system, the enzymatic activity was only restored to approximately 45% compared to PPC without PCMB treatment, which was used as a control. With increasing cysteine concentration, the enzymatic activity gradually increased, and when the concentration of cysteine was 8 mM, the enzymatic activity was approximately the same as that of the control. Because cysteine alone has no significant influence on PP and PPC activity (Supporting Information, Figure S4), the recovery of PPC activity was caused by the removal of the PCMB molecule in the protein upon cysteine treatment. Moreover, these results suggest that regulating PPC

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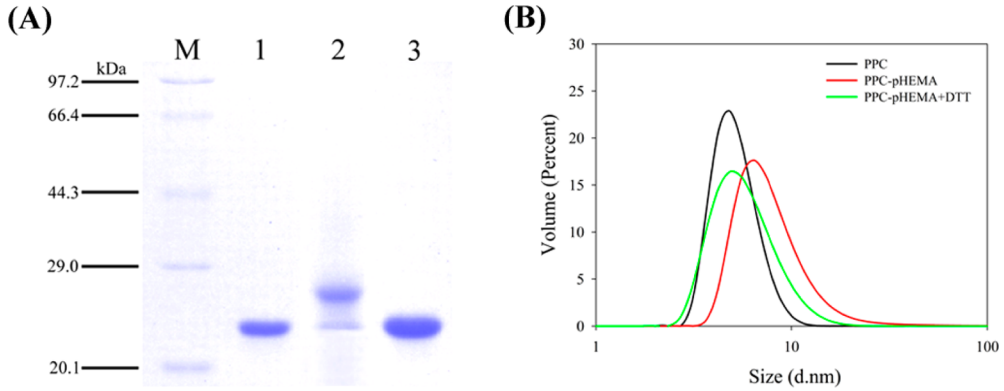

Figure 2. SDS-PAGE analysis of pHEMA conjugation (A) and hydrodynamic size distributions as determined by DLS (B). Lane M, standard protein marker. Lane 1, PPC. Lane 2, PPC-pHEMA. Lane 3, PPC-pHEMA + DTT.

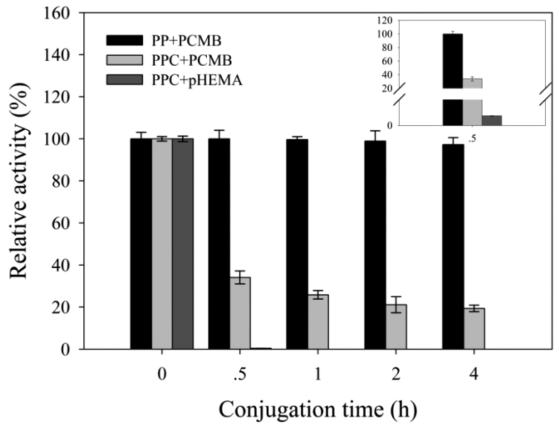

Figure 3. Influence of PCMB and pHEMA on the PPi hydrolysis activity of PP and PPC.

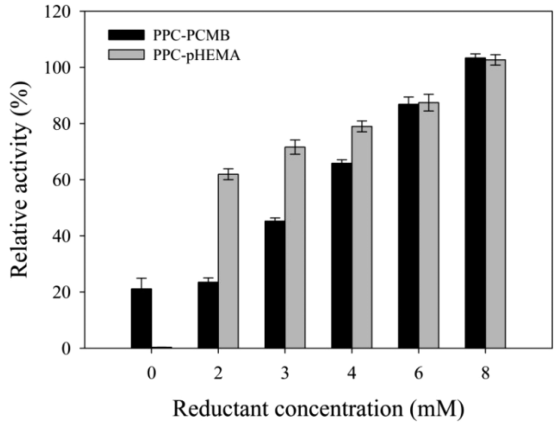

Figure 4. Recovery of the enzymatic activity of PPC−PCMB and PPC−pHEMA conjugates by different concentrations of reductants. Here, a relative activity of 100 corresponds to the activity of PPC without treatment.

activity can be achieved by altering the cysteine concentration in the reaction system.

Meanwhile, the activity of PPC-pHEMA was gradually recovered with increasing DTT concentration (Figure 4). The enzymatic activity was restored to 62% compared to PPC without treatment when the DTT concentration was 2 mM, and then restored to the same level of the control when the DTT concentration was 8 mM. To exclude the influence of DTT on protein activity, the activity of PP and PPC with different concentrations of DTT was measured and the results show that the DTT has no obvious effect on PP and PPC activity (Supporting Information, Figure S5). Therefore, the recovery of the activity was caused by the removal of the conjugated pHEMA from the protein upon DTT reduction, and the alteration of protein activity from "off" to "on" was realized by DTT treatment of the pHEMA conjugated PPC.

The above results demonstrate that the inhibitory effect of PCMB/pHEMA conjugation on protein activity can be gradually recovered by reductive reagents at different concentrations.

Reversible Regulation of Protein Activity by the PCMB/Cysteine and pHEMA/DTT Systems. Reversible regulation of protein activity is directly related to the application of smart systems in enzyme engineering, bioreactors, and biosensors. Therefore, the mechanism by which the reversible control of proteins is attained, particularly enzyme activity in practical applications, is an important research area.

To achieve reversible regulation of the PPC activity, we conducted a series of studies with the PCMB/cysteine and pHEMA/DTT systems. The enzymatic activity of PPC was first inhibited by PCMB and pHEMA conjugation and subsequently completely recovered by the addition of reductive reagents. The protein was then treated with PCMB and pHEMA again, and the PCMB and pHEMA maintained the inhibitory effect on the enzymatic activity, similar to the first treatment. Moreover, the activity of the PPC inhibited by PCMB and pHEMA was restored with reductant treatment (Figure 5). These results indicate that reversible regulation of the PPC can be easily achieved with PCMB/cysteine and pHEMA/DTT systems, and pHEMA/DTT can regulate the protein activity in a much larger range, compared with PCMB/cysteine. The mechanism of PPC in hydrolysis of sodium pyrophosphate before and after modifications is analyzed by Michaelis−Menten kinetics. The results show that PCMB and pHEMA conjugation have little influence on the substrate affinity (K_M) of PPC (Supporting Information Table S3). However, the turnover numbers (k_{cat}) of the two conjugates significantly decreased compared with that of the nonconjugated PPC, leading to an obvious decrease of catalytic efficiency (k_{cat}/K_M). Moreover, pHEMA has a more remarkable effect on the catalytic efficiency than small molecule PCMB in protein conjugation. Additionally, in both cases the change of k_{cat} and k_{cat}/K_M can be recovered largely by reductant treatment.

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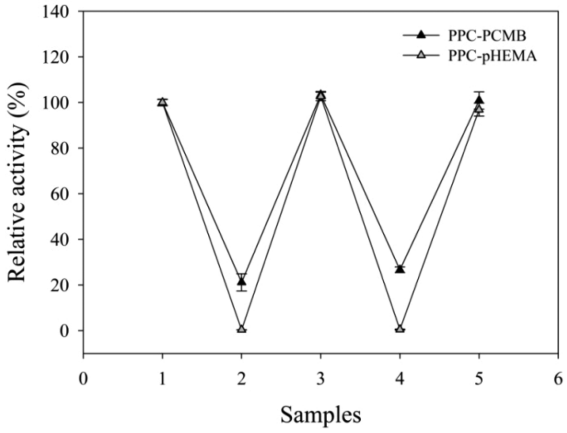

Figure 5. Repeatability of PPC activity regulation by PCMB and pHEMA. Sample 1, PPC without treatment. Sample 2, PCMB and pHEMA modified PPC. Sample 3, Reductant treated PPC−PCMB and PPC−pHEMA. Sample 4, Retreated sample 3 with PCMB and pHEMA. Sample 5, Reductant treated Sample 4. Here, the relative activity of 100 corresponds to the activity of the PPC without treatment.

Influence of PCMB and pHEMA Conjugation on Protein Conformation. Conformational changes of the PPC treated with PCMB/cysteine and pHEMA/DTT were determined by CD to determine the mechanism of protein activity change caused by the sulfhydryl group in the protein with different modifications. The CD signal from PPC in the 190−250 nm region was almost unchanged (Figure 6A and C) but the CD signal in the 250−350 nm region was significantly altered (Figure 6B and D) before and after PCMB/cysteine and pHEMA/DTT treatment, suggesting that the PCMB/cysteine and pHEMA/DTT treatment did not affect the secondary structure of PPC but showed a significant effect on the tertiary structure of PPC.

The CD spectrum of the PPC in the far ultraviolet region revealed a positive band at 292 nm and a negative band at 278 nm (Figure 6B). The modification by PCMB caused a decrease in the intensity of the band at 278 nm, indicating that the tertiary structure of PPC was changed in a certain degree by PCMB modification. Cysteine-treated PPC−PCMB caused enhancement of the CD band at 278 nm, and the signal intensity was nearly the same as that of the protein without treatment (Figure 6B). The conjugation of pHEMA caused a much larger decrease in the intensity of the band at 292 and 278 nm, indicating that the tertiary structure of PPC was significantly changed by pHEMA conjugation (Figure 6D). DTT-treated PPC-pHEMA caused a recovery of the CD band at 292 and 278 nm, and the signal intensity was nearly restored to the same level as the protein without treatment (Figure 6D).

For the effect of the PCMB and pHEMA conjugation on protein activity, the tertiary structure of PPC was changed when the sulfhydryl group in the Cys residue was modified by PCMB

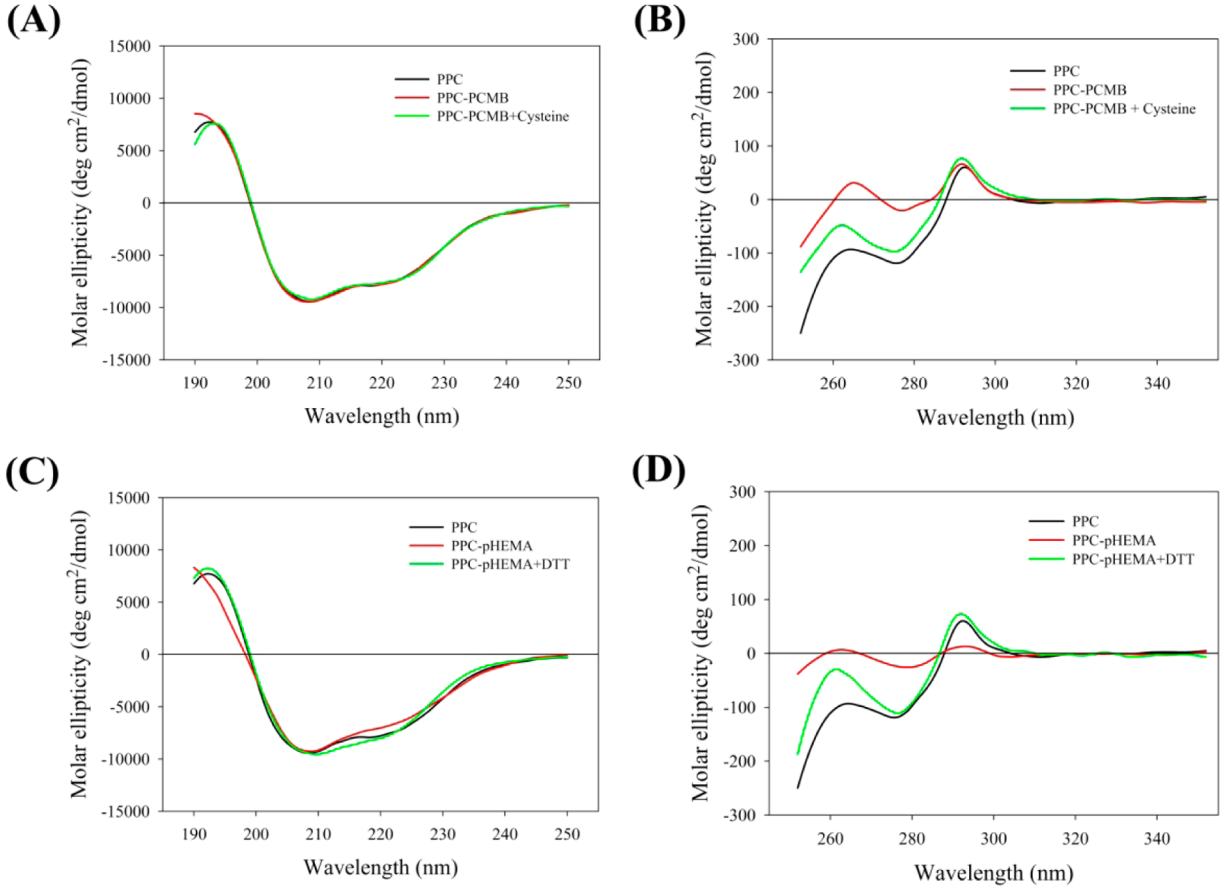

Figure 6. CD spectra in the near-ultraviolet region (A and C) and far ultraviolet region (B and D) of PPC with different chemical treatments. PPC, PPC without treatment. PPC−PCMB, PCMB modified PPC. PPC−PCMB+Cysteine, Cysteine-treated PPC−PCMB. PPC−pHEMA, pHEMA modified PPC. PPC−pHEMA+DTT, DTT-treated PPC−pHEMA.

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and pHEMA, and its activity greatly decreased. After reductant treatment, the modified sulfhydryl group was restored to its original reduction state; therefore, the tertiary structure of the PPC was restored to the original conformation and its activity was greatly recovered. The effect of PCMB on tertiary structure may be due to the benzoic acid group in the protein introduced by the PCMB modification of the sulfhydryl group, which has a different polarity and side chain length and other chemical properties from the sulfhydryl group.[39] This leads to a subtle change in the local conformation near the active site of the protein, which eventually has a considerable effect on the protein activity. In addition, the significant change of tertiary structure caused by pHEMA conjugation may be due to the steric blocking effect on the active center of the protein, which finally exerts a great effect on protein activity.

The above studies demonstrate that the effect of PCMB and pHEMA conjugation and removal of the modification by reductive reagents on the tertiary structure of PPC was the primary reason for activity regulation. The larger size of the pHEMA chain can exert significant influence on the tertiary structure of the protein, and leads to the obvious inhibitory effect on protein activity, compared with small molecule PCMB conjugation.

■ CONCLUSION

We illustrated that the introduction of a Cys residue into an appropriate position on the surface of the PPase near the active center offers the opportunity for protein activity control at different levels. A sulfhydryl group of the newly introduced Cys residue on the variant PPC surface provided a specific target for conjugation of sulfhydryl modifier PCMB and pHEMA, leading to a significant decrease in enzymatic activity, and the protein activity can be restored to different extents the treatment with different amounts of reductive reagents. Furthermore, CD measurement determined that the effect of PCMB and pHEMA on PPase activity was caused by a conformational change of protein. Compared with the effect of small molecule modification (PCMB), the pHEMA conjugation led to greater inhibitory effect on protein activity due to the significant change of the tertiary structure of PPC after conjugation, and the recovery of protein activity by reductive reagents was caused by the return of protein conformation, resulting from the dissociation between PPC and PCMB or pHEMA. Moreover, the PPC activity can be reversibly regulated by alternately repeated PCMB/pHEMA conjugation and reductant treatment. We expected that this study illustrated a new method for efficient regulating protein activity, and the strategy involved can be potentially generalized to be suitable for other protein systems.

■ MATERIALS AND METHODS

Chemicals. DNA polymerase (PrimeStar HS), restriction endonucleases, and T4 DNA ligase were purchased from Takara Biotechnology Co. Pfu DNA polymerase (Fermentas) was obtained from Thermo Fisher Scientific Inc. Oligonucleotides used as the PCR primers were synthesized at Sangon Biotech (Shanghai) Co., Ltd. Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid) was purchased from Sigma Chemical Co. *p*-Chloromercuribenzoate and cysteine were purchased from Sangon Biotech (Shanghai) Co., Ltd. Copper(I) bromide (CuBr, Fluka, 98%) was purified by stirring in acetic acid, washing with methanol, and drying in a vacuum. 2-Hydroxyethyl methacrylate (HEMA, Acros, 98%) was distilled under reduced pressure to remove inhibitors. 2,2-Bipyridyl (Bpy, Sigma, 99%) and the other reagents were used as received.

Generation of PPase Cys Mutant. *Bacterial Strains, Plasmids, and Culture Conditions.* *E. coli* K-12 was used as the wild-type strain and *E. coli* XLI-Blue was used as the host for gene cloning and protein expression of the native and mutant PPases. The plasmid pQE30 was used as the vector for the cloning of *ppa* gene and production of the native and mutant PPases. All of the *E. coli* strains were grown in either liquid LB medium (Luria nutrient medium consisting of 1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl, pH 7.0) or on LB plates (LB medium containing 1.5% agar). Ampicillin (final concentration: 0.1 mg/mL) and tetracycline hydrochloride (final concentration: 0.025 mg/mL) were added when required.

Cloning of the ppa Gene. Polymerase chain reaction (PCR) was used for the cloning of *ppa* gene encoding the *E. coli* PPase. The PCR template was the chromosomal DNA isolated from *E. coli* K-12, and the primers used for the reaction were 5′-CGCGGATCCAGCTTACTCAACGTCCCT-3′ and 5′-CGCAAGCTTTTATTTATTCTTTGCGCGCTC-3′. The PCR product was digested with *Bam*HI and *Hin*dIII and ligated into the *Bam*HI-*Hin*dIII site of the pQE30 vector. The recombinant plasmids were transformed into *E. coli* XLI-Blue, and the positive clones containing the *ppa* gene were screened for the production of *E. coli* PPase. The PCR was performed in a thermal cycler (MasterCycler ep gradient S, Eppendorf, Germany).

Site-Directed Mutagenesis. Site-directed mutagenesis was performed according to the megaprimer PCR method.[40−42] The forward flanking primer sequence used in the megaprimer PCR was 5′-CGCAAGCTTTTATTTATTCTTTGCGC-GCTC-3′, and the reverse flanking primer sequence was 5′-CGCGGATCCAGCTTACTCAACGTCCCT-3′. The mutagenic primer used to create the K148C-PPase mutant was 5′-CCTCGAAAAAGGCTGCTGGGTGAAAGTTGAAGG-3′ (the underlined nucleotides are the mutated codons). The megaprimer PCR products were digested with *Bam*HI and *Hin*dIII and then ligated into the *Bam*HI-*Hin*dIII site of the pQE30 vector as described previously. DNA sequencing was performed to verify the mutation (Shanghai Sangon Biotech Co., Ltd., China).

Expression and Purification. An overnight culture of *E. coli* XLI-Blue cells expressing the wild-type and mutant PPases were inoculated in liquid LB medium at 1:100 dilution, and then incubated on a shaker at 37 °C until it reached an OD_{600} of 0.5. IPTG (final concentration: 0.5 mM) was added and the cultures were incubated with shaking at 37 °C for 3 h to induce the expression of PPases. The cells were pelleted by centrifugation and the obtained cell precipitates were disrupted with lysozyme and sonication in 50 mM phosphate buffer (pH 8.0). After removing the cell debris by centrifugation, the PPases in the supernatant were purified over Ni-NTA Sepharose Resin (Shanghai Sangon Biotech Co., Ltd., China) according to the manufacturer's instructions and then concentrated using centrifuge filters (Amicon Millipore with 50 kDa molecular weight cutoff). The purity of the protein was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed using a 4% stacking and a 12% separating gel in a Mini-Protein II

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apparatus (Bio-Rad), and the gels were stained with Coomassie Brilliant Blue and examined with an EC3 imaging system.

Synthesis of Pyridyl Disulfide-Functionalized Polymer. Pyridyl disulfide-functionalized initiator was synthesized and used for CuBr/bpy-mediated atom transfer radical polymerization (ATRP) of HEMA according to literature procedure.[35] Briefly, HEMA (1.0 mL, 8.24 mmol), CuBr (24 mg, 0.168 mmol), and bpy (52 mg, 0.332 mmol) were dissolved in methanol (1.0 mL). The solution was purged for 30 min with nitrogen to remove the oxygen. Degassed pyridyl disulfide-functionalized initiator (58 mg, 0.164 mmol) was added to start the reaction in a glovebox under nitrogen purge. The mixture was stirred at room temperature for 90 min. To stop the reaction, the polymerization solution was diluted with methanol and filtered over neutral aluminum oxide column. The polymers were isolated by precipitation in cold diethyl ether. Precipitation was repeated 4 times. ^{1}H NMR spectra of the polymers were recorded on an INOVA 400 MHz nuclear magnetic resonance instrument, using CD_3OD as solvent. The molecular weights and molecular weight distributions of the polymers were measured on an Agilent PL-GPC 50 gel permeation chromatography (GPC) equipped with a refractive index detector, using a 5 μm Guard and 5 μm MIXED-D column with PMMA standard samples, and 0.05 mol/L lithium bromide solution in DMF was used as the eluent at a flow rate of 0.8 mL/min operated at 50 °C.

Conjugation of PCMB to PPC. PCMB modification of the Cys residues in the PPC was performed at 40 °C in 50 mM Tris-HCl buffer (pH 8.0) containing 0.05 mM PPC and 3 mM PCMB. Sodium pyrophosphate (2 mM) was added at different times to determine the remaining activity. Protection and reactivation of the PPC−PCMB was performed at 40 °C for 1 h by adding Cys (2−8 mM) after PCMB modification. The unreacted PCMB and Cys molecules were removed by centrifugation at 7500 *g* for 10 min. The process was carried out at 4 °C in 30 kDa molecular weight cutoff centrifuge filters (Amicon Millipore) at least 5 times.

Conjugation of pHEMA to PPC. 15.0 μmol of pyridyl disulfide-functionalized pHEMA was dissolved in 4.5 mL of methanol, and dropped into a PPC solution (0.3 μmol in 5 mL of phosphate buffer, pH 8.0). The mixture was incubated for 30 min at room temperature. A small aliquot was immediately used for SDS-PAGE analysis after evaporation of the methanol. The remaining solution was dialyzed (MWCO = 8000−14000 Da), lyophilized, and used for protein activity assay. Cleaving of polymer from the PPC-pHEMA conjugate was performed at 40 °C for 2 h by adding DTT (2−8 mM). The unreacted DTT molecules were removed by centrifugation at 7500 *g* for 10 min. The process was carried out at 4 °C in 30 kDa molecular weight cutoff centrifuge filters (Amicon Millipore) for at least 5 times.

Determination of Free Thiol Content. Free thiol content was determined using Ellman's assay.[43,44] 5,5′-Dithio-bis(2-nitrobenzoic acid) (Ellman's reagent, 4.0 mg, 0.010 mmol) was dissolved in 1 mL sodium phosphate buffer (PB, pH 8.0, 0.1 M) containing 1 mM EDTA to prepare the Ellman's reagent solution. A 250 μL aliquot of the protein sample (4.0 mg/mL), 50 μL Ellman's reagent solution, and 2.50 mL PB were mixed for 20 min at room temperature. The absorbance at 412 nm was measured with a spectrophotometer (Varioskan Flash, Thermo Scientific, USA). The thiol concentration was calculated using Beer−Lambert's law, with a molar extinction coefficient for 2-nitro-5-thiobenzoic acid = 14150 M^{-1} cm^{-1} at 412 nm.

Dynamic Light Scattering Measurement. The size analysis was performed by dynamic light scattering (DLS) measurements using Zetasizer Nano-ZS90 (Malvern Instrument Ltd. UK) at room temperature.

PPase Activity Assay. The PPase activity of the samples was assayed using the method of Heinonen and Lahti.[45] Briefly, enzymatic hydrolysis of sodium pyrophosphate by PPase was performed at 30 °C for 10 min in 50 mM Tris-HCl buffer (pH 8.0) containing 0.2−3.5 μg/mL PPase, 50 mM $MgCl_2$, and 2 mM PPi. The reaction (V = 100 μL) was terminated by the addition of 10 μL 0.4 M citric acid, and then 800 μL AAM solution (acetone-acid-molybdate) was added to the tubes. The contents were mixed and 80 μL 1 M citric acid was added. After mixing again, the yellow color was measured with a spectrophotometer at 355 nm. AAM solution was prepared daily by mixing 1 volume 10 mM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ with 1 volume 2.5 M H_2SO_4 and 2 volumes acetone. The protein concentration was determined using the method of Bradford[46] with bovine serum albumin as the protein standard. Michaelis−Menten parameters (K_M and k_{cat}) of sodium pyrophosphate hydrolysis were determined based on Lineweaver−Burk plot.[47] Briefly, the initial hydrolysis rate (V_0) was measured with an increase in substrate concentration ($[S]$) and determined from the time curves. Plotting $1/V_0$ versus $1/[S]$ yields a straight line, and the K_M and V_{max} were obtained according to the vertical intercept and horizontal intercept. k_{cat} was calculated by the equation of $V_{max} = k_{cat}$ [E], where [E] refers to enzyme concentration.

Circular Dichroism Measurements. The CD spectra were obtained on an AVIV 410 Circular Dichroism Spectrometer (AVIV Biomedical, Inc. Lakewood, NJ USA) at room temperature with a 0.4 cm quartz cuvette. The protein samples were dissolved in 50 mM Tris-HCl buffer (pH 8.0) at a protein concentration of 0.02 mg/mL measured from 190 to 250 nm and a concentration of 1.5 mg/mL measured from 250 to 350 nm. Each CD spectrum was the average of three scans, with a scan rate of 20 nm/min. Background scans without protein in solution were obtained in the same condition and subtracted from the wavelength scans prior to converting the millidegrees to the mean residue molar ellipticity.

■ ASSOCIATED CONTENT

Supporting Information

DNA sequencing of wild-type *ppa* gene and mutant *ppa* gene, metal ion selectivity of the PP and PPC, ^{1}H NMR spectrum and GPC trace of the pyridyl disulfide-functionalized pHEMA, CD spectra of the PP and PPC, influence of cysteine and DTT on the PPi hydrolysis activity of the PP and PPC, effect of pHEMA with different molecular weights on PPC activity, and Michaelis−Menten parameters of sodium pyrophosphate hydrolysis of PPC, the conjugated products and reduced products. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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