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Acta Biomaterialia 1 (2005) 183–191
Acta BIOMATERIALIA
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A stable three enzyme creatinine biosensor. 2. Analysis of the impact of silver ions on creatine amidinohydrolase

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Received 11 October 2004; received in revised form 26 November 2004; accepted 28 November 2004

Abstract

The enzyme creatine amidinohydrolase is a clinically important enzyme used in the determination of creatinine in blood and urine. Continuous use biosensors are becoming more important in the clinical setting; however, long-use creatinine biosensors have not been commercialized due to the complexity of the three-enzyme creatinine biosensor and the lack of stability of its components. This paper, the second in a series of three, describes the immobilization and stabilization of creatine amidinohydrolase. Creatine amidinohydrolase modified with poly(ethylene glycol) activated with isocyanate retains significant activity after modification. The enzyme was successfully immobilized into hydrophilic polyurethanes using a reactive prepolymer strategy. The immobilized enzyme retained significant activity over a 30 day period at 37 °C and was irreversibly immobilized into the polymer. Despite being stabilized in the polymer, the enzyme remained highly sensitive to silver ions which were released from the amperometric electrodes. Computational analysis of the structure of the protein using the Gaussian network model suggests that the silver ions bind tightly to a cysteine residue preventing normal enzyme dynamics and catalysis.

Keywords: Biosensor; Creatinine; Polyurethane; Creatine amidinohydrolase; Immobilization

1. Introduction

Clinical analyzers require biosensors to be used repeatedly while in contact with biological fluids (blood or urine) [1,2]. These systems often require a biosensor to operate continuously at 37 °C for more than one week (greater than 500 samples) [3]. For many biosensors, the stability of the enzyme layer limits the practical lifetime of the sensor. Our interest lies in the stabilization of enzymes via multi-point covalent immobilization in polyurethanes [4–6]. We hypothesize that the significant stabilization that can be imparted via incorporation into polyurethanes will enable the construction of enzyme-containing layers capable of multiple use in amperometric biosensors.

The detection of creatinine levels in biological fluids is an increasingly important clinical goal. This analyte is used for the evaluation of renal function and muscle damage. There has been considerable interest in the design of biosensors specific for creatinine. A three-enzyme biosensor has been developed that converts creatinine to amperometrically measurable hydrogen peroxide (H_2O_2) [7]. Due to the complexity of the three-enzyme system, the development and commercialization of the creatinine biosensor has been slow [8]. A number of

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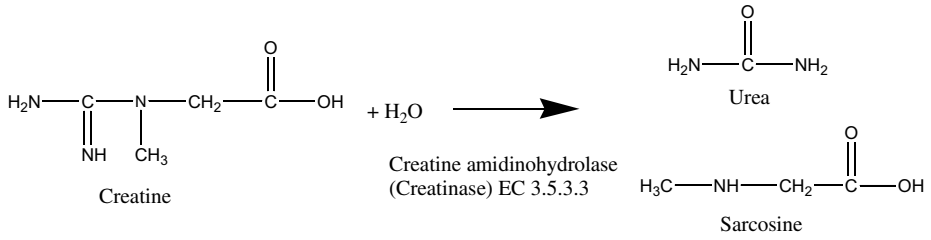
1742-7061/$ - see front matter © 2004 Published by Elsevier Ltd. on behalf of Acta Materialia Inc.
doi:10.1016/j.actbio.2004.11.007



Scheme 1. Creatine amidinohydrolase catalyzed hydrolysis of creatine.

methods are reported in the literature for the immobilization of enzymes for use in creatinine biosensors and the method of immobilization is the most important factor in the determination of the operational and storage stability of the biosensor [8].

Creatine amidinohydrolase (creatinase, EC 3.5.3.3) is a homodimer with subunit molecular weights of approximately 45 kDa. The enzyme catalyzes the hydrolysis of creatine (see Scheme 1). The two active sites of the protein are at the interface of the monomers being shared by each monomer and only the dimer is active. The enzyme has a low functional stability [9]. Additives such as reducing agents, proteins and polyols have been shown to increase the stability of the enzyme [9]. The low intrinsic stability of creatine amidinohydrolase has prompted the use of protein engineering to improve stability [10]. Herein we describe the modification, immobilization and stabilization of creatine amidinohydrolase.

2. Materials and methods

2.1. Materials

Creatine amidinohydrolase (from *Actinobacillus* sp., CRH-211) and sarcosine oxidase (from *Arthrobacter* sp., SAO-341) were purchased from Toyobo Co., Ltd. All enzymes were used without further purification. Poly(ethylene glycol) activated with isocyanate (mPEG-NCO) (Mw 5000) was obtained from Shearwater Polymers Inc. (Huntsville, AL). Hypol 2060G prepolymer, a toluene diisocyanate based prepolymer [11], was purchased from Hampshire Chemical (Lexington, MA). All other reagents were purchased from Sigma-Aldrich Chemicals (St. Louis, MO) and were of the highest purity available.

2.2. PEGylation of creatine amidinohydrolase

PEG-NCO was added at room temperature to a buffered solution (50 mM phosphate, pH 7.5) containing 3 mg/ml creatine amidinohydrolase. The ratio of PEG-NCO to enzyme was adjusted from 0:1 to 100:1. The reaction was mixed for 30 min, followed by overnight dialysis against 50 mM phosphate buffer at 4 °C [12]. Enzyme activity following modification was determined using an end point assay (described below).

2.3. Synthesis of creatine amidinohydrolase-containing polyurethane hydrogel

Hypol prepolymer 2060G (0.4 g) was added to a buffered solution (3.6 g of 50 mM phosphate buffer, pH 7.5) containing creatine amidinohydrolase (0–100 units enzyme per gram of prepolymer). The aqueous polymer solution was mixed for 30 s in a weigh boat until the onset of gelation. Polymerization was very rapid and gelation was usually complete within 1 min.

2.4. Characterization of enzyme modification

MALDI-MS analyses were performed using a Perspective Biosystems Voyager Elite MALDI-TOF. The acceleration voltage was set to 20 kV in a linear mode. One microlitres of PEGylated enzyme solution (0.1 mg/ml) was mixed with 1 μl of matrix solution (0.5 ml water, 0.5 ml of acetonitrile, 1 μl of trifluoracetic acid, and 10 mg of sinapinic acid). Spectra were recorded after evaporation of the solvent mixture and were calibrated externally using equine cytochrome C (12,361.96 Da), rabbit muscle aldolase (39,212.28 Da) and bovine serum albumin (66,430.09 Da).

2.5. End point assay for creatine amidinohydrolase activity

Creatine amidinohydrolase activity was monitored using a colorimetric assay that measures urea formation from the hydrolysis of creatine [7]. An enzyme solution (0.1 ml) was incubated with a mixture (0.90 ml) of 100 mM creatine in sodium phosphate buffer (50 mM, pH 7.5) at 37 °C for 10 min. The reaction was stopped by adding 2.0 ml of a solution containing Ehrlich's reagent (2.0 g of *p*-dimethyl aminobenzaldehyde in 100 ml of dimethyl sulfoxide plus 15 ml of concentrated HCl). The solution was incubated for 20 min at room temperature and the absorbance at 435 nm was measured. One unit was defined as the amount of enzyme

that catalyzes the hydrolysis of 1 μmol of substrate per minute.

2.6. Oxygen monitor assay for creatine amidinohydrolase activity

The initial rate of oxygen consumption was measured at 37 °C with a Clark oxygen electrode from Yellow Springs Instruments (Yellow Springs, OH). The reaction was initiated by adding an enzyme solution (1 μl) or an enzyme-containing polymer (10–100 mg cut into small pieces) to 5.0 ml of substrate (100 mM creatine in 50 mM phosphate buffer, pH 7.5 with at least 6 U/ml sarcosine oxidase). Before measurement, the assay solution was allowed to equilibrate to 37 °C with air. Oxygen consumption was measured for 5–10 min.

2.7. Thermostability creatine amidinohydrolase

Thermoinactivation of native and PEG-creatine amidinohydrolase was monitored at 37 °C in buffer (50 mM sodium phosphate, 2 mM EDTA, pH 7.5). The enzyme concentration in all samples was 0.08–0.1 mg/ml. Samples were removed periodically and assayed for activity using the end point assay.

Enzyme polymer samples were cut into small pieces and added to buffer (50 mM phosphate, pH 7.5) and incubated at 37 °C. Samples were removed periodically and assayed for enzymatic activity using the oxygen electrode.

2.8. Inhibition of creatine amidinohydrolase by silver ions

Creatine amidinohydrolase (1.0 mg/ml) was incubated in 20 mM Tris–HCl (pH 7.5) with silver nitrate (0–1 mM) at room temperature. Samples were removed periodically and assayed using the end point assay. In order to test if creatine acted as a competitive inhibitor for silver inhibition, the end point assay was performed as above with silver nitrate added to the creatine solution (0–100 μM $AgNO_3$ in 20 mM Tris buffer). After 10 min of incubation, the stop solution (*p*-dimethyl benzaldehyde solution) was added. Control experiments showed silver nitrate had no effect on the color development.

2.9. The use of additives to prevent silver inhibition of creatine amidinohydrolase

Creatine amidinohydrolase (1.0 mg/ml) was prepared in solution with either 50 mM EDTA, 50 mM EGTA, 50 mM mercaptoethanol, 50 mM DTT or 50 mM cysteine with 20 mM Tris, pH 7.5. Silver nitrate was added to give a final concentration of 0–100 μM. After the addition of silver nitrate, the solutions were allowed to incubate for 5 min and residual activity was determined using the end point assay.

2.10. UV–Vis absorption spectra of creatine amidinohydrolase

UV spectra were collected as previously described [13]. In short, creatine amidinohydrolase (1 mg/ml) was dissolved in 20 mM Tris (pH 7.5) with varying concentrations of silver nitrate (0–100 μM). In quartz cuvettes, the UV absorption spectra of the enzyme solutions were collected from 230 to 300 nm. Difference spectra were obtained by subtracting the spectra of the native enzyme (no silver) from the silver-ion-containing spectra.

2.11. Selection of a suitable template for GNM analysis

The crystal structure of *Actinobacillus* creatinase (Protein data base (PDB) code: 1KP0) was used to analyze collective dynamics via the Gaussian network model (GNM) [14]. The CMS (carbamoyl sarcosine, a creatine analog) bound form of *A. Bacillus* creatinase was used in order to determine the location of the residues in the active site. The CMS bound *A. Bacillus* creatinase structure was computationally modeled by combining the CMS molecule in *P. putida* creatinase structure (PDB code: 1CHM) with the *A. Bacillus* creatinase structure, followed by standard energy minimization using the Molecular Operating Environment package (MOE) [15]. The local energy minimization algorithms of MOE apply nonlinear function optimization to the potential energy function to determine a nearby conformation for which the forces on the atoms are zero [16].

3. Results and discussion

3.1. Effect of PEGylation on enzyme activity and stability

Prior to covalent immobilization, we were interested in determining the effect of chemical modification on creatine amidinohydrolase activity and stability. PEGylation accurately mimics the first steps in polyurethane-based immobilization strategies and we therefore PEGylated the enzyme. Creatine amidinohydrolase can be modified with reactive PEGs to a high degree without significant loss of enzyme activity (data not shown). Modification of each monomer of the enzyme with an average of 5 PEG chains led to a loss of only 30% activity.

Although much of the enzyme activity was retained after modification, a significant decrease in enzyme stability was observed when the modified enzyme was stored in buffer at 37 °C (Fig. 1). Native enzyme loses

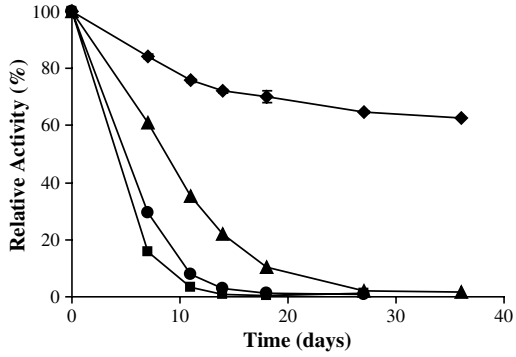


Fig. 1. Stability of creatine amidinohydrolase as a function of number of PEGs attached at 37 °C. Native enzyme (closed diamonds); one PEG attached (closed triangles); three PEGs attached (closed circles); five PEGs attached (closed squares).

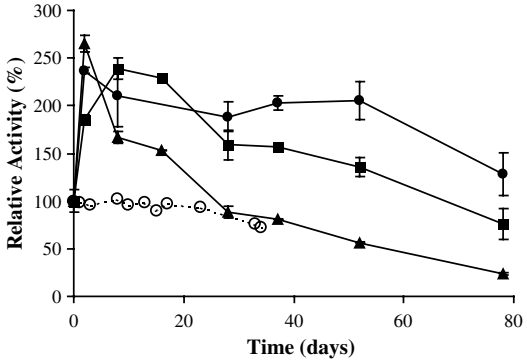


Fig. 2. Stability of creatine amidinohydrolase-containing polyurethane hydrogels stored in buffer at 37 °C. Rates were normalized to the rate of oxygen consumption after the first day. Native enzyme (open circles); Immobilized enzyme: 10 units/g polymer (closed triangles); 50 units/g polymer (closed squares); 100 units/g polymer (closed circles).

less than 40% activity in buffer at 37 °C over 40 days; however, the modified enzyme showed a substantial decrease in stability in solution. Since creatine amidinohydrolase is a homodimer with a low intrinsic stability [9] it is possible that chemical modification induces unfolding in this delicate protein. Although PEGylation often predicts the impact of polyurethane immobilization on activity, the stabilization of proteins by immobilization can be significantly different than the impact of PEGylation.

3.2. Immobilization of creatine amidinohydrolase in polyurethane polymers

Biopolymers were prepared with enzyme concentrations of 0–100 units/g of polymer. The specific activities of the enzyme–polymers were directly proportional to enzyme concentration (data not shown). This implies that the rates measured under these conditions are not diffusion-controlled and reflect only the enzymatic reaction rate [17]. The average activity retention for creatine amidinohydrolase in the polyurethane polymers was 28%.

The immobilized polymers were tested for reusability by repeatedly assaying a single gel sample for eight cycles with no loss of apparent activity (data not shown). As seen with other polyurethane chemistries, the enzyme was well immobilized within the gel and no leaching of enzyme from the polymer to the surrounding solvent was observed [4–6].

The storage stability of the enzyme in the polymer was determined by incubating enzyme–polymer samples in buffer at 37 °C and removing samples periodically to be assayed (Fig. 2). Interestingly, the activity of the polymerized enzyme increased during the first week of storage. This increase in activity may be due to a change in polymer properties although the effect was not observed with related enzymes in these polymers. After the first week, the enzyme activity began to decrease and the rate of loss in observed enzyme activity was dependent on the concentration of the enzyme in the polymer. Polymers with high enzyme content showed slower deactivation. Nonetheless, significant activity was retained in all polymers for up to 80 days which is more than long enough for use in a diagnostic biosensor. The stability reduction caused by PEGylation was not observed in the immobilized enzyme. Given that only upon immobilization is the enzyme "locked" into a fixed conformation these data support an intuitive expectation of stability enhancements.

3.3. Effect of silver on enzyme activity

Before being used in a functioning sensor, polyurethane-immobilized enzyme must be applied to amperometric electrodes that contain Ag/AgCl. Given the known propensity of silver ions to interact with proteins [3,18], the effect of silver ions on enzyme activity was explored. Silver-induced inactivation becomes more of a concern if a sensor will be used in series with other sensors for a long period of time since the enzyme will have significant time to scavenge silver from solution. The enzyme was incubated with 5–100 μM silver nitrate and assayed for residual creatine amidinohydrolase activity (Fig. 3). Silver is a very effective inhibitor of creatine amidinohydrolase, completely inhibiting enzyme activity even when the silver concentration is on the same order as the enzyme concentration.

In order to determine whether silver was acting as a competitive inhibitor, differing concentrations of silver and 90 mM creatine were added to the buffer. The enzyme (~0.7 μM) was added directly to this solution and after 10 min the concentration of urea in the system was determined (data not shown). The presence of creatine did not slow enzyme inhibition. Silver must therefore be binding at a location other than the active site.

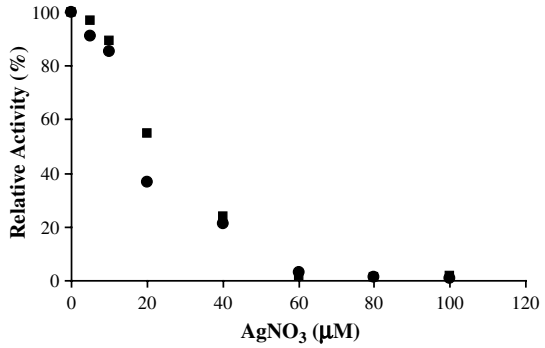


Fig. 3. Silver-induced deactivation of creatine amidinohydrolase in solution. Incubation time: 5 min (closed circles); 15 min (closed squares).

It is important to note that the inactivation was not reversible. Dilution of the inhibited enzyme did not regenerate activity and nor did dialysis overnight with either EDTA or DTT (metal chelating agents). This suggests that silver is bound tightly to the enzyme and is not likely to dissociate.

Since the enzyme is effectively inhibited by silver ions at concentrations that could be released from electrodes in a clinical biosensor, it is of interest to develop methods to prevent or at least slow enzyme inhibition by silver. Silver can be effectively scavenged by a number of chelating agents. We investigated using 50 mM solutions of EDTA, EGTA, DTT, mercaptoethanol, cysteine, imidazole and polyethyleneimine (PEI) to sequester silver. Inhibition by silver ions was effectively prevented by pre-incubation with thiol containing compounds (cysteine, mercaptoethanol, DTT) and prevented somewhat by PEI (Fig. 4). Hence, thiol-containing compounds may be effective in scavenging silver ions that leach from the electrode and may be useful in preventing enzyme deactivation.

UV/Vis spectroscopy was used to determine if any major structural changes occurred upon binding of silver to the enzyme. An increase in absorbance at

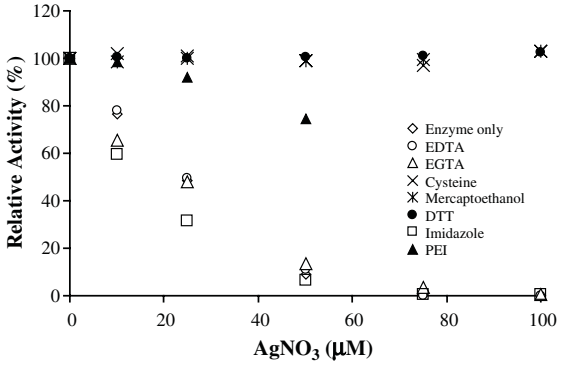


Fig. 4. Protection of creatine amidinohydrolase from silver-induced inactivation.

247 nm was apparent with increasing concentrations of silver. This is likely due to the formation of ligand-to-metal charge transition (LMCT) bands. LMCT bands caused by charge transitions that occur when ligands in proteins bind to metal centers, such as Ag (I) [13]. Using Raman spectroscopy, Shen et al. showed that silver ions formed covalent bonds with sulfur atoms in human serum albumin (HSA) [13]. Since Ag (I) is a soft Lewis acid, it should have a high affinity for the soft donor sulfur atom [19] and hence one can expect strong interactions between the cysteine residues of creatine amidinohydrolase and silver.

Coll et al. [20] and Yoshimoto et al. [21] have shown that the modification of sulfhydryl groups on creatine amidinohydrolase causes complete loss of activity. This is interesting since the Cys residues are distant from the active site and have no known role in catalysis [20,22]. The loss of activity has been attributed to the possible prevention of domain motions by alkylation, thus locking the enzyme into an inactive conformation [20].

3.4. Collective dynamics of creatine amidinohydrolase

To further investigate how Cys residues, and their interaction with silver ions, might contribute to the function and stability of creatine amidinohydrolase, the dynamics of the protein were examined with the GNM [14]. The GNM is an elastic network model that has been shown to predict the collective dynamics of proteins. The model results are in close agreement with the temperature (B) factors from X-ray crystallographic experiments [14,23] and the free energy costs of H/D exchange [24]. This approach permits us to decompose conformational motions into a series of orthogonal modes, ranked by their associated frequencies. The modes with the slowest frequency (the slowest mode), also referred to as 'global' motions, usually reveal the functional movements that engage the entire molecule [24–32]. The fastest modes, on the other hand, indicate 'local' motions, and point to individual residues involved in the early folding/stabilization process [28,32]. The major utility of the GNM is its efficient applicability to the dynamics of large structures (such as creatine amidinohydrolase, a dimer of $N = 804$ residues) that are beyond the range of molecular dynamics simulations.

The mean-square fluctuations of creatine amidinohydrolase residues predicted by the GNM were compared with the experimental B factors [33] (Fig. 5(a)). The results are displayed for only one monomer since both monomers show almost identical fluctuation behavior. The monomer structure is composed of two lobes, N-lobe and C-lobe at the N- and C-termini, composed of 160 and 240 residues, respectively. The N-terminal end shows the highest fluctuations. The close agreement between theoretical and experimental values further validates the use of the GNM model. Fig. 5(b) displays

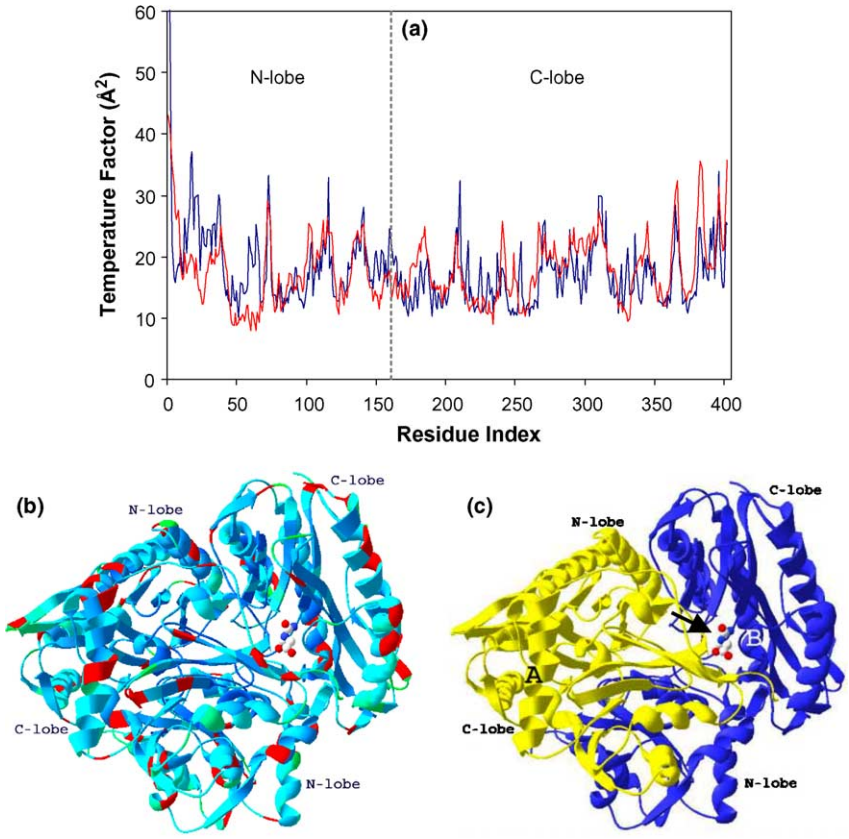


Fig. 5. Residue fluctuations in creatinase. (a) Comparison of predicted (by GNM; shown in blue) and experimental (X-ray crystallographic; red) B-factors. (b) Color-coded ribbon diagram of the monomer showing the most flexible regions (peaks in a) in red, and the least flexible (minima) in blue. (c) Ribbon diagram illustrating the relative positions of the monomers A and B with respect to the ligand, CMS, which is indicated by a black arrow. (For interpretation of color in this figure, the reader is referred to the web version of this article.)

the ribbon diagram of the enzyme, color-coded from red to blue, [2] in the order of decreasing mean-square-fluctuations. Fig. 5(c) shows the location of the two monomers and the substrate analog (CMS).

Next, we focused on the global motion modes to infer information on functional dynamics. Fig. 6 shows the mobilities of residues in the most representative global mode of the enzyme, which effectively displays the demarcation between the two lobes. The ordinate scales with the square displacements of the individual residues. Peaks indicate the most mobile regions, and minima refer to the global hinge (or anchoring) regions, about which the concerted motions of large substructures (domains, subunits, etc.) take place. The residues involved in the catalytic activity of the enzyme (shown by the open blue circles) typically exhibit minimal fluctuations. This type of mechanical constraint near the catalytic site is consistent with the fine-tuning and cooperativity of enzymatic activity, as observed in other enzymes [34]. We note that the substrate analog (CMS) is not symmetrically bound with respect to the two monomers in the

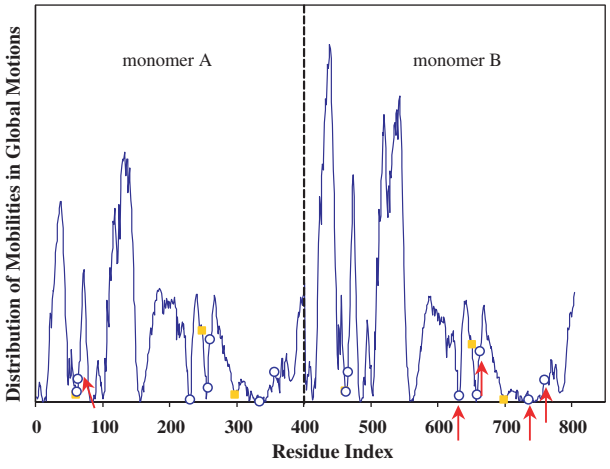


Fig. 6. Distribution of mobilities in the dominant global mode of creatinase dimer. The catalytic residues Phe62, Arg64, His231, Tyr257, Glu261, Arg 334 and Glu357 are shown by the blue open circles and Cys60, Cys249 and Cys297 are shown by orange squares, in both monomers (separated by the dashed line). Red arrows indicate the catalytic residues that coordinate the CMS in the examined crystal structure. (For interpretation of color in this figure, the reader is referred to the web version of this article.)

[2] For interpretation of color in Figs. 5–8, the reader is referred to the web version of this article.

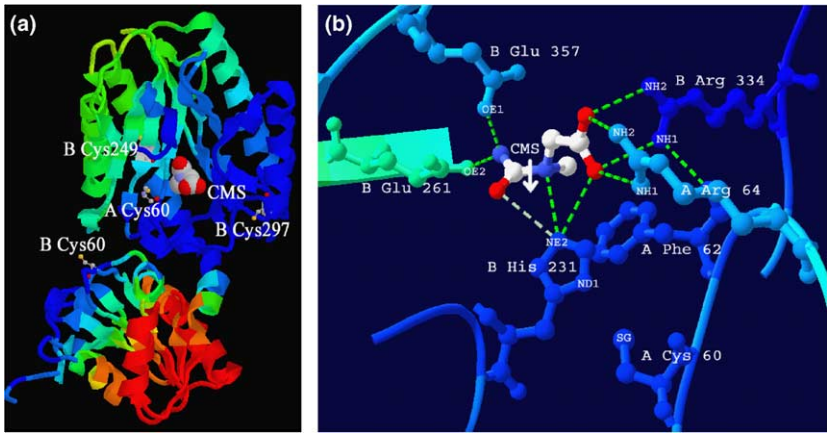


Fig. 7. (a) Color-coded ribbon diagram illustrating the mobility of creatinase residues in global motions. The colors blue–green–yellow–orange–red are used in the order of increasing mobility. Creatine analog, CMS, is shown in space-filling representation. The yellow arrows indicate the three cysteines (shown in ball-and-stick) on monomer B, and the white arrow the Cys60 on monomer A. Atoms in CMS and cysteines are colored by CPK convention. (b) Dynamics near the active site. Creatine analog, CMS, and key catalytic residues are shown in ball-and-stick. Catalytic residues' sidechains and their associated backbone (ribbon) are colored according to their global mobilities (same as (a), shown from a different perspective for clarity). Hydrogen bonds are shown in dashed lines. The location of the peptide bond cleavage during catalytic electron transfer is shown by the white arrow. The moderate mobility of Glu261 and Glu357, which form hydrogen bonds with the nitrogen atom in the guanidine group of CMS may facilitate the peptide bond cleavage. Phe62 and Arg64 join the catalytic pocket from the other chain. (For interpretation of color in this figure, the reader is referred to the web version of this article.)

PDB structure, but interacts more closely with one of the chains (monomer B) and subset of catalytic residues (shown by arrows in Fig. 6). That said, both chains exhibit the same global dynamics, and thus are equally disposed to bind the substrate.

The N-terminal lobe exhibits the largest amplitude motions. Cys60 and Cys297 are both located in highly constrained hinge/anchoring regions on the respective C- and N-lobes (Fig. 6). In particular Cys297 resides in a highly stable central region near the interface between the two lobes, and plays a dual role in controlling domain and sub-domain motions (evidenced by the minima observed at this residue in the dominant modes). The N-terminal domain serves as a concertedly moving lid that allows the catalytic pocket in the C-terminal domain of the other chain to be exposed to solvent and to recruit the substrate inside. Cys60 belonging to the A chain closely coordinates the substrate, and is also distinguished by severely constrained and highly cooperative dynamics (minima in Fig. 6).

Fig. 7 provides a closer view of the catalytic binding pocket. The oxygen atoms $O^{\varepsilon 1}$ of Glu357 and $O^{\varepsilon 2}$ of Glu261 on monomer B form hydrogen bonds with a nitrogen atom in the guanidine group of CMS (Fig. 7), which may facilitate the peptide bond breakage between the guanidine group and the resultant sarcosine molecule. Likewise, the residues Cys60, Phe62 and Arg64 on monomer A are spatially close to the B-monomer His231. His231 is the most critical residue that dictates the biocatalytic chemistry of creatine degradation [20,33]. His231 forms three hydrogen bonds with the substrate (shown by dashed lines), but not with any other amino acid. The sulfide atom (S^{γ}) on Cys60 could form a 'hydrogen-bond like' interaction with the nitrogen $N^{\delta 1}$ on His 231, which could assist the His231 side chain in stabilizing a catalytically potent conformation.

The folding nuclei were inferred from the high frequency modes predicted by the GNM (Fig. 8). Among the three cysteine residues, we note that Cys297 is distinguished by a peak in Fig. 8. Cys297 can be predicted, therefore, to be one of the most critical residues that are involved in the early folding process in creatine amidinohydrolase. We also observe a peak near Cys249.

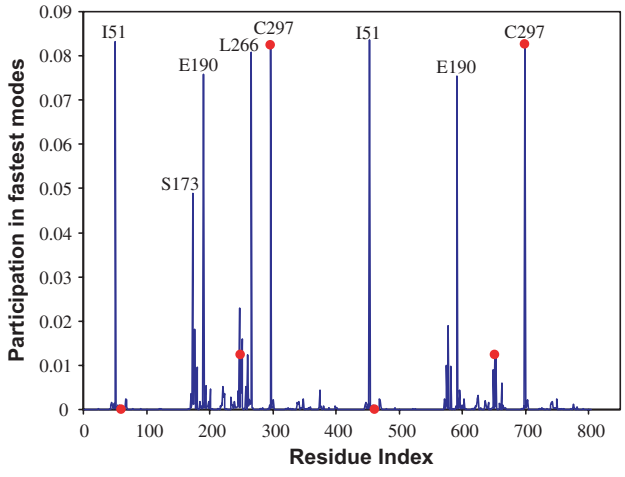


Fig. 8. Fluctuations in the high frequency modes. Peaks (labeled by the residue types and numbers) reveal the centers of localization of energy subject to the highest frequency vibrations. Cys60, Cys249 and Cys297 are shown by red circles. The highest peak at Cys297 suggests that this residue significantly contributes to folding/stability. (For interpretation of color in this figure, the reader is referred to the web version of this article.)

Cys249 is located in the folding core composed of three β-sheets (β9, β11 and β12) and part of two α-helices (α7 and α8) in a pita bread fold of C-terminal lobe. From the electrostatic analysis, we also found that Cys297 is surrounded by negatively charged patches (data not shown). We hypothesize that these patches preferentially recruit silver ions to Cys297, which is both structurally and functionally critical.

Obviously, silver binds strongly with creatine amidinohydrolase and completely inhibits enzyme function even at low concentrations. As reported in the other papers in this series, all three enzymes needed for the creatinine biosensor are inhibited to some extent by silver; thus, methods for preventing or slowing enzyme inactivation by silver are important.

4. Conclusions

We have shown that creatine amidinohydrolase can be effectively modified by PEG-NCO and covalently incorporated into polyurethane materials via modification by isocyanate prepolymers. Modification by increasing amounts of PEG appears to destabilize the enzyme leading to a decreased half-life when stored in buffer at room temperature. Creatine amidinohydrolase was successfully and irreversibly immobilized by chemical crosslinking into polyurethane polymers and retained significant activity (28%). Enzyme in the polymers was more than sufficiently stable to be used for 30 days in a buffered solution at 37 °C. Creatine amidinohydrolase is highly susceptible to inhibition by silver. Inhibition does not appear to be caused by a perturbation at the active site. Addition of silver scavenging thiol-containing molecules was effective in preventing the loss of enzyme activity due to silver. Spectroscopic studies suggest that metal-to-thiolate interactions may exist. Through the GNM analysis of the molecular dynamics of creatine amidinohydrolase, we were able to predict that two of the cysteine residues, Cys60 and Cys297 in both chains were critically important in controlling the functional dynamics of the enzyme. Furthermore, the analysis of high frequency motions suggest, that, if modified, these two sites may destabilize the enzyme and lead to inactivation. The fact that Cys297 is surrounded by negatively charged residues gives further support to the potential attraction of positively charged silver near this residue. The use of creatine amidinohydrolase in a biosensor will require protection from silver ions.

Acknowledgment

We gratefully acknowledge support provided by Bayer Diagnostics, Inc.

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