Published in final edited form as:

J Biomol Struct Dyn. 2015 ; 33(12): 2619–2632. doi:10.1080/07391102.2014.1003196.

Caseoperoxidase, Mixed β -Casein-SDS-Hemin-Imidazole Complex: A Nano Artificial Enzyme

Zainab Moosavi-Movahedi¹, Hussein Gharibi², Hamid Hadi-Alijanvand³, Mohammad Akbarzadeh³, Mansoor Esmaili³, Maliheh S. Atri³, Yahya Sefidbakht³, Mousa Bohlooli³, Khodadad Nazari⁴, Soheila Javadian², Jun Hong⁵, Ali A. Saboury^{3,6}, Nader Sheibani⁷, and Ali A. Moosavi-Movahedi^{3,6,*}

¹Chemistry & Chemical Engineering Research Center of Iran (CCERCI), Tehran, Iran

²Department of Chemistry, Faculty of Science, Tarbiat Modares University, Tehran, Iran

³Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran

⁴Research Institute of Petroleum Industry, Tehran, Iran

⁵College of Life Science, Henan University, Kaifeng, 475000, China

⁶Center of Excellence in Biothermodynamics, University of Tehran, Tehran, Iran

⁷Departments of Ophthalmology and Visual Sciences, Biomedical Engineering, and McPherson Eye Research Institute, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA

Abstract

A novel peroxidase-like artificial enzyme, named “caseoperoxidase”, was biomimetically designed using a nano artificial amino acid apo-protein hydrophobic pocket. This four-component nano artificial enzyme containing heme-imidazole- β -casein-SDS exhibited high activity growth and k_{cat} performance towards the native horseradish peroxidase (HRP) demonstrated by the steady state kinetics using UV-Vis spectrophotometry. The hydrophobicity and secondary structure of the caseoperoxidase were studied by ANS fluorescence and circular dichroism spectroscopy. Camel β -casein (C β -casein), with a flexible structure and exalted hydrophobicity, was selected as an appropriate apo-protein for the heme active site using a homology modeling method. Heme docking into the newly obtained C β -casein structure indicated one heme was mainly incorporated with C β -casein. The presence of a main electrostatic site for the active site in the C β -casein was also confirmed by experimental methods through Wyman binding potential and isothermal titration calorimetry. The existence of C β -casein protein in this biocatalyst lowered the suicide inactivation, and indicated that the obtained structure has a good protective role for the heme active-site. Additional further experiments confirmed the retention of caseoperoxidase structure and function as an artificial enzyme.

*Corresponding author: Ali A. Moosavi-Movahedi, PhD, Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran, Tel: +9821 - 66403957, Fax: +9821 - 66404680, moosavi@ut.ac.ir.

Keywords

Caseoperoxidase; Biomimetic; Biocompatible; Artificial enzyme; Horseradish peroxidase (HRP); Camel β -casein; SDS monomer; Imidazole; Heme

1. Introduction

The term biomimetic chemistry describes a novel chemistry that is inspired by a living system (Breslow 2005; Marchetti & Levine 2011). Artificial enzymes can be considered a part of biomimetic chemistry in which the catalytic processes occur in living systems and not just as substances in biology. Biomimetic chemistry attempts to improve the performance of chemical reactions and catalysts by imitating enzymatic processes (Yatsimirsky 2004; Silavi, Divsalar & Saboury 2012). Robust biocatalysts may be formed by encapsulating a prosthetic group in hydrophobic pocket-like micelles (Moosavi-Movahedi et al. 2008; Wang et al. 2010), vesicles (Murakami, Hisaeda & Ohno 1984; Murakami, Hisaeda & Ohno 1987; Murakami, Hisaeda & Ohno 1990; Murakami, Hisaeda & Ohno 1991) and macrocyclic compounds (Bender & Komiyama 1978; D'Souza & M. L. Bender 1978; Tabushi & Yamamura 1983; Diederich 1988; Murakami, Kikuchi, Hisaeda & Hayashida 1996) that mimic the polypeptide envelope protecting the catalytic center of the natural enzymes. Artificial enzymes could be biomimetically constructed from native proteins as host for prosthetic active sites to simulate the catalytic functions exhibited by natural enzymes (Quilez, Lauzon, Desfosses, Mansuy & Mahy 1996; Grinbergs, O'Brien & Hrkal 1999; Monzani et al. 2001).

The heme group plays a fundamental role in the activation of hemoproteins and hemoenzymes, such as horseradish peroxidase (HRP). The heme moiety is protected by the hydrophobic surrounding to avoid the reduction of heme groups into inactive oxo-complexes. Our previous studies designed and constructed various peroxidase-like artificial enzymes based on several effective HRP existed parameters. These included the existence of axial histidinated heme complex (Moosavi-Movahedi et al. 2008), proper hydrophobic environment surrounding the heme-based active site (as micelles (Moosavi-Movahedi et al. 2008), revise micelles (Farivar et al. 2010), or assembled nafion (Hong et al. 2012)), or the existence of negative and positive surface charged hydrophobic pockets (vesicular peroxidase structure (Gharibi, Moosavi-Movahedi, Javadian, Nazari & Moosavi-Movahedi 2011)). The electronic properties and biochemical functions of heme prosthetic groups in hemoproteins strongly depend on the nature of the protein environment surrounding the heme group (Antonini & Brunori 1971; Wüthrich 1973; Mazumdar & Mitra 1993). The design of biomimetic protein pockets similar to those present in hemoproteins and heme-based enzymes is a new approach in the current study, and has recently maintained a high level of interest.

Caseins are one of the most important class of milk proteins consisting of α_{s1} -, α_{s2} -, β - and κ -caseins, which exist in proportions of approximately 4:1:4:1 by weight (Swaigood 1992; Barzegar et al. 2008). Bovine milk contains about 80% casein, of which 35% is β -casein (Swaigood 1992). Bovine β -casein is a small phosphoprotein with an open structure, and a

molecular mass of 23.58 kDa containing 209 amino acids (Carles, Huet & Dumas 1998). Camel β -Casein (C β -casein) is slightly bigger than bovine β -casein, with a molecular mass of 24.65 kDa containing 217 amino acids (Kappeler, Farah & Puhani 1998). Sequence alignment of bovine and camel β -casein indicated that the sequence similarity and identity between these two caseins are 84.5% and 67.2%, respectively (scheme 1) (Barzegar et al. 2008).

The structure of β -casein proteins is neither random coil nor regularly folded globular, and is very mobile in solution. Under physiological conditions caseins are classified in the Natively Unfolded (NU) (Farrell, Qi & Uversky 2006) or Intrinsically Unstructured Protein (IUP) groups (P.Tompa 2002). They are known for their highly flexible and non-compacted structure, as well as their transient or permanent binding to several different (mostly hydrophobic) ligands (Farrell et al. 2006). Like many other members of NU protein class, β -casein contains significant amounts of proline and glutamine (15% compared to the normal average 5%) (Yousefi et al. ; Farrell et al. 2006). These differences partially explain the low rigid structure of β -casein in aqueous solutions (Vicsizs, Szollosi, Friedrich & Tompa 2006). There are two entirely natural variants or forms of β -casein in the milk of dairy cows, normally called A1 and A2. The difference between A1 and A2 β -casein is in a single amino acid at position 67, histidine in A1 and proline in A2. Camel, goat and sheep all produce β -casein of the A2 type (Jinsmaa & Yoshikawa 1999).

Here we designed an efficient biocompatible artificial enzyme by embedding the heme-imidazole complex inside a hydrophobic pocket containing C β -casein-SDS, which mimicked the native apo-protein environment of HRP with good evolution towards the native peroxidase.

2. Materials and Methods

2.1. Materials

Bovine hemin, sodium dodecyl sulfate (SDS) and imidazole were obtained from Sigma (St. Louis, MO USA). Camel milk was generously provided by the Faculty of Veterinary, University of Tehran, Iran. Camel β -casein was purified according to modified methods (Rasmussen, Due & Petersen 1995; Egito et al. 2002). The purity of C β -casein was assessed using SDS-PAGE and estimated to be greater than 95%. Other chemicals were of analytical grade (Merck, Germany) and used without further purification. Hemin, hydrogen peroxide, and guaiacol solutions were freshly prepared. Absorption spectra were collected using a model Shimadzu- 3100 spectrophotometer with 1 cm path length cells equipped with a thermostatted holder. The steady state kinetics of guaiacol oxidation by hydrogen peroxide, catalyzed by the model catalyst, were monitored at 470 nm (colored product of the reaction) in 5 mM phosphate buffer solution (PBS) pH 7.

2.2. Methods

2.2.1. Steady state kinetics—The steady state kinetics of guaiacol (a hydrogen donor) oxidation by hydrogen peroxide, catalyzed by the artificial peroxidase enzyme, were obtained at 470 nm (colored product of the reaction) (Moosavi-Movahedi et al. 2008) in

PBS. Progress curves for the reactions were obtained at various guaiacol concentrations, and the initial rates were used to construct the Michaelis-Menten curves. The concentration of H_2O_2 was kept high (1.5 mM) and constant with respect to the hydrogen donor during the course of the reaction ensuring a pseudo first order kinetic. Steady state conditions were reached after a lag time of 7s. The concentration of hydrogen peroxide was determined from the absorbance measurements at 240 nm, with $\epsilon^{240} = 43.6 \text{ cm}^{-1} \text{ M}^{-1}$ (Moosavi-Movahedi et al. 2008).

2.2.2. Suicide inactivation—Suicide inactivation of the peroxidase-like artificial enzyme was monitored at 470 nm (λ_{max} for the product of the catalytic reaction) by difference spectrophotometry. The details of the procedures for measuring the product concentration, rate of reaction, and progress curves have been described previously. (Ghademarzi & Moosavi-Movahedi 1999; Khosraneh, Mahmoudi, Rahimi, Nazari & Moosavi-Movahedi 2007) Progress curves were determined by following the absorbance of the reaction mixture at 470 nm during a time course of 11.2 min. The unreacted hydrogen donor or reductant substrate (AH) concentration was calculated from the absorbance measurements and suitable relations and equations. The maximum increase in absorbance (A_{∞}) and the value of the intact activity (α_i) were determined by “End-point procedure” experiments. (Khosraneh et al. 2007) In the end-point method, an excess of fresh HRP solution was used in the incubated mixture. A_{∞} was used to calculate the concentration of unreacted AH ($[\text{AH}]_{\infty}$), and the intact activity of the enzyme (α_i). The intact activity (α_i) was defined as the enzyme activity before reacting with the substrate.

2.2.3. Circular dichroism (CD)—The CD spectra of the samples were recorded on a Jasco J-810 spectrophotometer equipped with a thermoelectric sample holder. Changes in the secondary structures were monitored in the far-UV region (190–260 nm) using a 1-mm path length. The baseline spectrum of the buffer was subtracted from each spectrum, and resultant values were expressed in mean residue ellipticity $[\theta]$ ($^{\circ} \text{ cm}^2 \text{ dmol}^{-1}$) based on a mean amino acid residue weight of 117.3 (MRW). The mean residue ellipticity was defined as $[\theta]_{\lambda} = (100\text{MRW}\theta/\text{cl})$, where θ is the observed ellipticity in degrees at a given wavelength, c is the protein or artificial enzyme concentration in mg/ml, and l is the length of the cell (cm).

2.2.4. ANS fluorescence—The 8-anilino-1-naphthalenesulfonic acid (ANS) was used as the hydrophobic probe to obtain the hydrophobicity of C β -casein and C β -casein-SDS. Fluorescence intensity measurements were performed using a Cary Eclipse spectrofluorimeter (Varian, Australia). All experiments were conducted using a 1-cm path length fluorescence cuvette and 5 nm slit size. The ANS binding assessments were carried out following incubation with 32.5 μM ANS for 5 min. During the fluorescence measurements of ANS–protein complex the wavelength of excitation was 390 nm while the emission spectra were recorded between 400 and 600 nm.

2.2.5. Binding Study

2.2.5.1 Spectroscopic methods: The binding of heme-imidazole complex to C β -casein-SDS was determined according to previous spectroscopic methods for binding dissection of

protein-ligand studies (Moosavi-Movahedi et al. 2006). The sample cell contained C β -casein (3.5 μ M) and SDS (0.9 mM), whereas the reference cell contained SDS (0.9 mM). heme-imidazole complex was added into both sample and reference cells to omit the absorbance interference of the free heme-imidazole and also the interaction of free SDS with heme-imidazole complex in each step of titration. It is important to note that at difference spectrophotometric titration methods (heme-imidazole complex, λ_{max} =413 nm), the absorbance interference of the C β -casein-SDS complex at 413 nm was ignored.

2.2.5.2 Calorimetric measurements: Isothermal titration calorimetric (ITC) experiments were carried out using a VP-ITC ultrasensitive titration calorimeter (NanoCal, LLC, North Hampton, MA). The nanocalorimeter consisted of a reference cell and a sample cell (1.8 mL in volume), with both cells insulated by an adiabatic shield. All solutions were thoroughly degassed before use by stirring under vacuum. The sample cell was loaded with SDS/C β -casein solution (0.96 mM/3.48 μ M), and the reference cell contained the buffer solution. The solution in the cell was stirred at 307 rpm using a syringe (equipped with a micropropeller) filled with heme/imidazole solution (175 μ M/ 52.5 mM) to ensure rapid mixing. Injections were started after the baseline stability was achieved. The titration of SDS/C β -casein with the heme/imidazole solution involved 30 consecutive injections of the ligand solution, with a first injection of 5 μ L and the remaining injections of 10 μ L. Each injection took 6 s, and they were introduced at 3 min intervals. The thermal effects due to SDS/C β -casein dilution were corrected based on control experiments in which aliquots of the buffer solution were injected into SDS/C β -casein solution. The enthalpy changes associated with processes occurring at a constant temperature were measured in ITC experiments. The measurements were performed at a constant temperature of 27.0°C controlled using a Poly-Science water bath.

2.2.6. Theoretical Methods—A proper template has not been found for C β -casein. The PSI-blast method was utilized to find a proper template for C β -casein. After repeating the process, several templates were found for the coverage of the whole C β -casein sequence instead of one template. The template sequence was selected based on the best PSI score to cover the majority of the β -casein sequence. The PDB ID of selected templates were 3FVW-B and 2XZL-A. To build accurate models, MODELLER 9.9 was used 1. The loop structures were optimized by using loop refinement routine of MODELLER. The best model was selected among 10,000 generated models based on Normalized DOPE z score. OpenMM Zephyr 0.9.2 used to perform short-MD simulation (Friedrichs et al. 2009). Casein structure was refined at room temperature by using AMBER96 force field and fat water model of water. The final structure of C β -casein model was utilized for docking using Hex 6 software (Ritchie, Kozakov & Vajda 2008). The binding energy between the heme and casein molecule was determined by VMD energy tool. (Humphrey, Dalke & Schulten 1996) The post processing stage of docking results was performed by using VEGA ZZ 2.3.2 (Pedretti, Villa & Vistoli 2002).

3. Results and Discussion

Previous studies indicated that in order to achieve an efficient peroxidase-like enzyme the heme group, as the peroxidase active site, should be protected by a suitable hydrophobic

pocket (Moosavi-Movahedi et al. 2008; Gharibi et al. 2011). The biomimetic is the design of a polypeptide pocket to achieve more resemblance across the native HRP. The more peroxidatic function could be expected by approaching the native peroxidase structure. A protein (C β -casein) with a hole inside, appropriate for the heme placing, could be chosen for this reason. Here the preference is to select a natively unstructured and flexible protein such as C β -casein, which adapts itself to cover the ligand heme as the peroxidase active-site. However, heme accommodation inside a rigid and structured protein with a probable unsized hole and inflexible structure is difficult. In the current study two approaches were taken to overcome such limitations: first a structural approach, and second a functional approach to generate a new artificial enzyme.

3.1. Theoretical modeling

To construct an appropriate peroxidase-like enzyme, it is necessary to protect the heme group as the peroxidase active-site via a hydrophobic pocket (Wang et al. 2007; Moosavi-Movahedi et al. 2008; Wang, Yang, Ma, Chang & Xu 2008; Gharibi et al. 2011). One of the biomimetic steps for more resemblance to native peroxidase is to prepare a polypeptide pocket as the hydrophobic environment. The C β -casein belongs to the family of Intrinsically Unstructured Protein (IUP) groups (P.Tompa 2002), and there is no x-ray structure for C β -casein in protein data bank. To better determine whether C β -casein has an appropriate apo-protein role for the heme prosthetic group, the homology modeling was performed to attain its structure. Figures 1 A and B show the final achievement of C β -casein (camel shape structure) as an optimized structure after several steps of MD.

After docking the optimized heme monomer structure to newly achieved C β -casein structure, the best structure among the population of Hex generated docked complexes (the maximum of population distribution) was extracted. These results indicated that C β -casein could accommodate the heme prosthetic group inside its cavity (Figure 1C). In addition, almost 1/3 of heme was placed into the protein hole, and the interaction of heme with the edge of apo-protein hole was electrostatic. The amino acid residues within four radius angstroms, near heme major site in C β -casein protein, were gln 56, asp 59, glu 60, gln 62, asp 63, lys 64, ile 65, val 75, his 78, thr 79, glu 80, ile 82 and pro 102 indicating that this site is among a set of hydrophobic (nonpolar) and electrostatic (polar) amino acids.

Additional docking studies of the heme into bovine β -casein were performed. The differences of binding energy for heme docking into bovine β -casein (-34 ± 2 kcal/mol) and C β -casein (-31 ± 5 kcal/mol) were not significant. However, there is a histidine residue that is far from heme (only 10 angstroms) in C β -casein (Figure 2), while there is no histidine around the heme site in bovine β -casein. We chose C β -casein for further experiments because of the histidine amino acid near the heme major site, which is essential for peroxidase activity.

Figure 3 shows the heme molecule in C β -casein docking analysis. Figure 3A shows the RMSD distribution or changed structure of bound heme- C β -casein relative to the structure of C β -casein alone, before heme binding. The value 1.1 for the RMSD in the maximum population was reasonable, and it meant that the 3D structure of C β -casein is not damaged after heme binding. In addition, the structure of C β -casein alone possesses the capacity of

heme molecule inclusion. Figure 3B demonstrates the accessible surface area (ASA) population distribution of protein after heme binding. We postulated that if the protein binding sites have equal preference in the ligand binding the distribution shape would be changed into a straight line. However, the presence of preferable binding sites for ligand on the protein created the distribution shape and the most population was due to its peak.

The asymmetric peak of Figure 3B indicates the presence of a hidden minor peak population (minor binding site) in addition to the major binding site. The protein dipole moment distribution, after heme binding (Figure 3C), indicates two binding sites or sets. One site with abundant population had lower value of dipole moment than the other site with low population. The charge of the protein and ligand was similar in all cases, thus, the dipole moment variation was due to the change in direction of ligand binding to the protein. However, the population peak indicated that one direction was preferred for binding of heme to C β -casein. Figure 3D shows the distribution of polar accessible surface area (PSA) of protein displaying the ASA and dipole moment effects simultaneously in the distribution, which confirmed the presence of major and minor heme binding sites in C β -casein.

3.2. Experimental studies

There are some experimental improvements to enhance the resemblance of this new peroxidase-like biocatalyst structure to native HRP. First, C β -casein cavity should grow and adapt itself to incorporate the heme prosthetic group. Furthermore, the heme complex that has a dimer structure under the aqueous experimental conditions, should convert into heme monomer complex. Thus, experimental improvement of the new peroxidase-like biocatalyst close to native HRP structure and activity were a further step towards constructing an efficient peroxidase-like artificial enzyme. To study and determine the structure of new peroxidase biocatalyst based on HRP, the peroxidatic function was examined for different designed complexes via reverse function to structure studies. Figure 4 shows the time course activity curves for different heme based biocatalysts. Figure 5 indicates the UV-Vis spectra for different peroxidase-like biocatalysts.

Although the low red shift in heme-C β -casein compared with the heme-only spectrum pointed out to hydrophobic interaction of heme and C β -casein, the Soret peak showed the dimer heme template (Figure 5A) (Shantha, Saini, Thanga & Verma 2001). Figure 4 shows that the heme-C β -casein did not reach a good peroxidase activity, and that the heme was not incorporated in to the C β -casein because of the dimmeric heme group against the small or undersized hole in C β -casein. The presence of an imidazole group, as peroxidase activator (Gharibi et al. 2011) and heme-monomerizing agent, helped with further placement of the heme into the protein and increased the activity slope compared with the similar biocatalysts without imidazole ligand (Figure 4). Figure 5A shows that the heme-imidazole was changed to monomerized heme template (Simplicio & Schwenzer 1973). Sodium dodecyl sulfate (SDS) almost activated the biocatalysts at levels below its micelle concentration. Figure 4 indicates that the four components C β -casein-SDS-heme-imidazole biocatalyst have the optimum activity by the inserted particular effects of C β -casein. The most proceeding enzyme template of C β -casein-SDS-heme-imidazole biocatalyst also indicated the

stabilizing roles of C β -casein and SDS next together compared to other artificial biocatalysts (Figure 4).

Figure 5B indicates that this four-component biocatalyst was most similar to HRP spectrum intensity or structure compared to other analogous biocatalysts. Thus, the four-component C β -casein-SDS-heme-imidazole biocatalyst with the best peroxidase activity and structure here was named Caseoperoxidase by us. The column curves in Figure 6 show the identification of the concentration of each sub-activator for optimum activity of caseoperoxidase. The best selected concentrations for optimum activity for caseoperoxidase were: heme (12 μ M), imidazole (3.6 mM), SDS (0.9 mM) and C β -casein (3.25 μ M).

The ANS-fluorescence experiments were performed to determine the SDS effects on C β -casein. The increased ANS-fluorescence emission of C β -casein-SDS relative to C β -casein alone (Figure 7A) indicated that more hydrophobic patches were exposed by SDS monomers (0.9 mM) (Haskard & Li-Chan 1998). Thus, in the presence of SDS the C β -casein monomer cavities have reached the appropriate size for heme-imidazole complex as the active-site.

Figure 7B shows the CD spectra for the secondary structure of C β -casein varying by SDS and heme groups. The decreased ellipticity θ at 222 nm in the presence of SDS, heme or both together near C β -casein, indicated the more stable C β -casein secondary structure (Hirst & III 1994) as a result of ordered C β -casein's α -helices. In the presence of imidazole, the spectra showed profound perturbations (not shown). Imidazole moiety cannot be used below 220 nm because of high absorbance at far UV CD region overwhelming the spectrum. Table 1 shows the analysis of CD spectra for secondary structure at 195–260 nm by using CDNN software. The presence of SDS with C β -casein resulted in an increase in the percentage of α -helix while decreasing β -sheet. These analyses also pointed to the more stable C β -casein-SDS secondary structure. Although C β -casein with or without SDS and heme did not exhibit a particular near CD spectra for tertiary structure, but the gain of a secondary structure by SDS/heme had paramount importance (not shown). It seems that heme operates as a bobbin or a nucleus that would structuralize the flexible and natively unfolded C β -casein (Figure 8). These results indicated that the presence of SDS monomer had a specific effect through its hydrophobic glue-type behavior to preserve or hold the heme species near the protein and support the newly achieved structure (figure 8).

3.3. The binding of heme-imidazole complex to C β -casein-SDS

Figure 9 shows the absorption changes at 413 nm versus the concentrations of heme-imidazole complex into C β -casein-SDS. There is one plateau indicating the possible existence of nearly one heme-imadazole complex binding set. It was proposed that at the saturation point the molar concentration of the protein–ligand complex was equal to the total molar concentration of the protein (Moosavi-Movahedi et al. 2006). Evidently, the molar concentration of the bound heme-imadazole complex at this point was n times greater than the molar concentration of the C β -casein– heme-imadazole complex (n is the maximum number of binding sites in the first binding set). Thus, dividing the absorbance difference at the saturation point by the total molar concentration of the protein multiplied by n resulted in the molar extinction coefficient of bound heme-imadazole complex binding set (ϵ^b_{413}). We calculated “ n ” (Figure 9) for the binding set by dividing the total molar concentration of

heme-imadazole complex by total C β -casein molar concentration (Figure 9B-inset) at the inflection point of the saturated phase. Therefore, n was equal to 4.76, thus indicating there are near five total binding sites on C β -casein-SDS for hemeimidazole ligand. In this way, ϵ^b_{413} for this binding set of the bound heme-imadazole complex was $17885.6 \text{ M}^{-1} \text{ cm}^{-1}$. The molar extinction of free heme-imadazole (ϵ^f_{413}) was $54996.6 \text{ M}^{-1} \text{ cm}^{-1}$ (Figure 9A-inset). The determination of the molar extinction coefficient for the bound hemeimidazole complexes enabled us to obtain the number of moles of heme-imadazole bound to one mole of protein (ν), which was necessary to draw a typical binding isotherm plot (Moosavi-Movahedi et al. 2006). It is worthy to define “ n ” as the total macromolecular interaction sites and ν as possible number of binding sites.

The mono-phase plot showed that there was approximately one set for binding heme-imidazole to C β -casein-SDS. The value ν (the number of moles of bound heme-imidazole per mole of C β -casein) could be find in each titration step according to the plot in Figure 9 (Moosavi-Movahedi et al. 2006). The Wyman binding potential, π , (Wyman 1965) could be calculated from the area under the binding isotherm according to Eq. 1.

$$\pi = RT \int_{\nu_i=0}^{\nu_i} \nu_i \partial \ln[hem-im]_f \quad (1)$$

Alternatively, the binding potential is due to the apparent binding constant (K_{app}) as follows (equation 2) (Jones & Manley 1982).

$$\pi = RT \ln(1 + K_{app} [hem-im]_f^{\nu_i}) \quad (2)$$

Possessing R as the universal gas constant, T as the temperature in Kelvin and π binding potential, the values of K_{app} as a function of ν could be determined by application of equation 2. G° and ΔG°_ν were achieved using equations 3 and 4.

$$\Delta G^\circ = -RT \ln K_{app} \quad (3)$$

$$\Delta G^\circ_\nu = \Delta G^\circ / \nu \quad (4)$$

Figure 10 is the plot of ΔG°_ν versus ν , and demonstrated the presence of one main, strong electrostatic binding site (the first phase on the plot), and approximately four-minor, weak non-electrostatic (hydrophobic) sites for heme-imidazole to C β -casein-SDS (the second phase on the plot), approaching $\Delta G^\circ_\nu=0$. These experimental achievements confirmed the results of the theoretical studies.

Calorimetric measurements according to heme binding to C β -casein-SDS were also performed to confirm the spectroscopic binding achievements. Figure 11 shows the calorimetric curves that indicated the interactions between heme-imidazole and C β -casein-SDS (3.48 μ M/0.96 mM). The curve indicated that the heat of each injection as a function of the heme-imidazole complex/C β -casein-SDS molar ratio, which could fit according to two binding set programs. As can be seen in calorimetric parameters summarized in Table 2, the

ΔH of the first binding set is -9.30×10^5 cal/mol, and the ΔH of the second binding set is -4.54×10^5 cal/mol. The hydrophobic interactions make endothermic contributions to the enthalpy of mixing, and the electrostatic interactions make exothermic contributions to the enthalpy (Moosavi-Movahedi et al. 2006). According to Table 2, both binding sets were associated with an exothermic net ΔH , indicating that the long-range electrostatic interactions between the charged regions of the heme-imidazole complexes (especially iron (III)) and the ionic regions of the protein/SDS were strong.

Figure 5 shows that the spectra of the heme complexes indicated the incorporation into the hydrophobic region. The heats of each binding set comprised of the sum of both electrostatic and hydrophobic interactions. However, the heat of the first binding set was much lower than that of the second one. Therefore, the first binding set with higher binding constant (K_b) associated with a more exothermic enthalpy (Table 2) corresponded to the electrostatic interactions of the heme-imidazole complex more closer to the charged regions of the protein/SDS. However, the lower exothermic ΔH corresponding to the second binding set (Table 2) was associated with the more hydrophobic interactions involved in accommodation of the heme-imidazole complex within the hydrophobic region of the protein/SDS as an apoprotein. Table 2 also indicates that the maximum number of heme-imidazole complexes that could be incorporated into each apo-enzyme (N) in the first and second binding set is about 4.6. Thus, the constructed biomacromolecule with multiple active-sites is a multi-enzyme. It is interesting that the justified ITC experiment did highly confirm the spectroscopic binding method because of similar and precise results of number of binding sets, the number of binding sites in each set, the binding preferences, and the types of interactions.

3.4. Peroxidatic activation of caseoperoxidase

Table 3 shows the kinetic parameters due to Michaelis-Menten constants (K_m , equation 5) for the various peroxidase-like biocatalyst models as a function of guaiacol concentration (Metzler 2003).

$$Rate = \frac{V_{max}[S]}{K_m + [S]} \quad (5)$$

The turnover number (catalytic rate constant, k_{cat}), which indicates the maximum moles of substrate converted to product per mole of catalyst per unit time, measured by the Lineweaver-Burk linearized Michaelis-Menten plot (Metzler 2003) (equations 6,7), is reported in Table 3.

$$\frac{1}{Rate} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (6)$$

$$k_{cat} = \frac{V_{max}}{[E]_t} \quad (7)$$

Table 3 shows k_{cat} , K_m , and catalytic efficiencies (k_{cat}/K_m) as the enzyme characteristics, as well as specific activities for the biocatalysts. In our models the reaction concentration of the artificial biocatalysts was different from the concentration of HRP, hence the catalyst performance needed to be quantified and compared by other parameters including k_{cat} or k_{cat}/K_m . Specific activity (under same conditions) could also be an appropriate parameter for comparison of biocatalysts because of their independence from enzyme concentration, and it indicates the same increasing trend as turnover number in biocatalysts (Table 3). Table 3 shows that C β -casein fortified the peroxidase-like enzyme as "caseoperoxidase". High activity growth (Figure 4 and Table 3) of caseoperoxidase was associated with the pioneered preparation of apo-protein (C β -casein-SDS) with a suitable hydrophobic environment, and imidazole complex to heme moiety as the representative of histidine amino-acid role's in native HRP active site.

In a previous study (Moosavi-Movahedi et al. 2008), it was demonstrated that the His-hematin-SDS (SDS was used at levels above the critical micelle concentration) combination had almost similar K_m and k_{cat} values (enzyme characteristics) as those reported here in the presence of C β -casein (Table 3). It seems that C β -casein at low concentration (3.25 μ M) in the current biocatalyst compensated for the role of SDS micelle (90 mM) (in His-hematin-SDS biocatalyst) with the hydrophobic interior and electrostatic outside as a model for artificial apo-protein for peroxidase. Thus, the enzyme characteristics are operating similarly. The preference of this new artificial enzyme is its enhanced biocompatibility compared to the surfactant vesicular and micellar biocatalysts.

3.5. Suicide inactivation

In the presence of high peroxide concentrations, peroxidase can also enter into a third pathway (inactivation), called "suicide inactivation" (Scheme 2). (Hosler 2004; Shang, Liu & Hu 2006; Gharibi et al. 2011) The whole forms of active artificial enzyme, inactivated artificial enzyme, hydrogen donor (reductant substrate), the catalytic reaction product (tetraguaiacol), and the apparent rate constant of inactivation are AE_a , AE_i , AH, P, and the parameter k_i , respectively (Scheme 2). (Gharibi et al. 2011) The decrease of the active enzymatic forms (free active artificial enzymes (AE_a), compound I (C-I), and compound II (C-II) (Khosraneh et al. 2007)) due to suicide inactivation could be estimated by monitoring the AH concentration during the catalytic reaction cycle. The rate of consumption of the reductant substrate (AH) and the overall rate of conversion of the active artificial enzyme (AE_a) to the inactivated form (AE_i) enabled us to obtain the differential kinetic equation as well as the integrated kinetic relation, according to previous models: (Khosraneh et al. 2007)

$$[AH]_t = [AH]_0 \times e^{\alpha_0/k_i(e^{-k_i \cdot t} - 1)} \quad (8)$$

$[AH]_0$ is the molar concentration of AH at $t=0$. Equation (8) describes the relationship between the concentrations of the remaining aromatic substrate $[AH]_t$ over time, taking into account the decrease in enzyme activity during the inactivation reaction. The kinetic suicide parameters α_0 and k_i could be determined using a nonlinear regression fit of the experimental data to the equation 8. For this purpose, commonly used computer software such as Excel Solver was utilized. The suicide kinetic parameters of the biocatalysts, obtained by fitting the experimental curves to Eq. (8) are summarized in Table 4. Figure 12 illustrates the accuracy of the kinetic model and the excellent agreement between experimental data and the curve fit (calculated) based on Eq. (8), indicating the similarity between the suicide mechanisms of the biocatalyst containing hemin-SDS-C β -casein-imidazole and HRP. The k_i for the hemin-imidazol-SDS-C β -casein catalyst was less than k_i for hemin-imidazol-SDS in 1.5 mM of H₂O₂, the common hydrogen peroxide concentration used here (Table 4). The α_0 parameters, indicating the presence of active enzyme in both biocatalysts are nearly similar (Table 4). These results indicated that the presence of C β -casein protein near SDS, both in monomer concentrations, improved the protection of the heme-imidazole group as an active site. Thus, C β -casein and SDS molecules, in particular concentrations, have a structured and resistant apo-enzyme pocket shielding heme catalytic center.

4. Conclusion

New four-component biocompatible nano-biocatalyst including C β -casein-SDS-heme-imidazole called here "caseoperoxidase" provided higher evolution of activity, specific activity, and catalytic efficiency toward native HRP. Caseoperoxidase is a biomimetic of native peroxidase that incorporates the active site by the protein's hydrophobic inside. The tertiary structure of C β -casein, not previously reported in protein data bank, was determined by a particular homology modeling method. C β -casein was selected as an appropriate apo-protein for the heme active site by homology modeling. Heme docking into the newly obtained C β -casein structure indicated that one heme incorporates with β -casein. Docking analysis predicted the presence of one main active site and some minor sites for the heme ligand in C β -Casein. The presence of one main electrostatic and nearly four minor non-electrostatic (hydrophobic) sites for the active-site into β -casein protein was also confirmed by experimental methods through Wyman binding potential and ITC.

The presence of SDS (monomer concentration) in caseoperoxidase had a particular supporting effect, which held the hydrophobic prosthetic active sites near C β -casein. In addition, it created an appropriate hydrophobic environment with more stabilized secondary structure of apo-protein incorporating the heme-imidazole active site inside. In addition, the presence of imidazole groups also further promoted the recent biocatalyst into an activated peroxidase-like biocatalyst as a mimic of native peroxidase. The apo-protein (C β -casein-SDS) was caused to be a receptor for approximately five heme-imidazole active sites. Thus, caseoperoxidase would be classified as a multi-enzyme model in aqueous solution. Loose

connection of prosthetic groups (heme-imidazole) into apo-enzyme facilitated the diffusion of aqueous substrate (like hydrogen peroxide and its derivatives) into the enzyme's inside pocket and constructed a dynamic multi-enzyme. Thus, Caseoperoxidase is an allegorical biocatalyst and set up cumulative inter and intra molecular interactions, and has the capability to structuralize a flexible, efficient and resistant multi-enzyme.

Acknowledgments

The support of University of Tehran, Iran National Science Foundation (INSF) and Iran National Elites Foundation (INEF), Iran Society of Biophysical Chemistry is gratefully acknowledged.

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Figure 1. The obtained final structure of C β -casein (camel shaped) from homology modeling steps: A) ribbon structure, B) structure with blue basic region, red acidic region, green polar region and grey non-polar region, C) the best resulted structure obtained from heme docked to C β -casein.

Figure 2.
The representative of histidine amino acid near heme bound to Camel β -casein (C β -casein)

Figure 3.

A) RMSD distribution or changed structure of bound heme- C β -Casein relative to the structure of C β -Casein alone, B) accessible surface area (ASA) population distribution of protein after heme binding, C) The protein dipole moment distribution after heme binding, D) the distribution of polar accessible surface area (PSA) of protein after heme binding

Figure 4.

The time course activity curves for different heme based biocatalysts, heme (12 μ M), imidazole (3 mM), SDS (0.9 mM) and C β -casein (3.25 μ M), HRP (3 nM), [H₂O₂] = 1.5 mM and [guaiacol] = 0.22 mM at 27°C

Figure 5. UV-Vis spectra for different peroxidase-like biocatalysts in two divisions A and B, the concentration of components are: heme 12 μ M, imidazole 3 mM, SDS 0.9 mM, C β -casein 3.25 μ M and HRP 12 μ M

Figure 6.

Identification of the best concentration of each sub-activator for optimum activity of caseoperoxidase. A) the biocatalyst: heme (12 μM), imidazole (3 mM), SDS (0.9 mM) and variation of Cβ-casein concentration, [H₂O₂] = 1.5 mM and [guaiacol] = 0.22 mM, B) the biocatalyst: heme (12 μM), imidazole (3 mM), Cβ-casein (3.25 μM) and variation of SDS concentration, [H₂O₂] = 1.5 mM and [guaiacol] = 0.22 mM, C) the biocatalyst: heme (12 μM), Cβ-casein (3.25 μM), SDS (0.9 mM) and variation of imidazole concentration, [H₂O₂] = 1.2 mM and [guaiacol] = 0.22 mM

Figure 7.

A) The fluorescence due to ANS for ANS alone, ANS-C β -casein and ANS-C β -casein-SDS at 27°C, the concentration of components are: C β -casein 3.25 μ M, SDS 0.9 mM and ANS 32.5 μ M **B)** CD spectra for the second structure of C β -casein protein, varying by SDS and heme agents, C β -casein (13.8 μ M), SDS (3.8 mM) and heme (51 μ M)

Figure 8. Schematic diagram showing the Heme/Imidazole active-sites in C β -casein-SDS hydrophobic pocket as a caseoperoxidase model.

Figure 9.

Main figure; the profile resulted from the difference absorbance titration (at 413 nm) of sample cell: C β -Casein (3.25 μM) –SDS (0.9 mM) and reference cell: SDS (0.9 mM) by insertion of 2 μl of heme/imidazole stock sample (0.6 mM/180 mM) each time in to sample and reference cells, versus the total concentration of heme-imidazole, Figure A inset; the variation of absorption at 413 nm due to heme-imidazole insertion into SDS solution (0.9 mM), the line slope is ϵ_f related to free heme-imidazole in SDS/buffer solution at 27°C, Figure B inset; the molar concentration of total β -casein protein as effect of dilution of the sample.

Figure 10.
The plot $\Delta G^\circ/w$ versus ν

Figure 11.

Calorimetric curves obtained from measurements of the heat of inserting heme_imidazole ($175 \mu\text{M}/52.5 \text{ mM}$) complexes into a 5 mM phosphate buffer, $\text{pH} = 7$, subtracted from the heat of inserting heme_imidazole ($175 \mu\text{M}/43.75 \text{ mM}$) complexes into C β -casein/ SDS ($3.48 \mu\text{M}/0.96 \text{ mM}$). The resulting heat was subtracted from the heat of inserting a 5 mM phosphate buffer into C β -casein/ SDS ($3.48 \mu\text{M}/0.96 \text{ mM}$). (A) The heat/s (heat power) of each injection as a function of time. (B) Heat of each injection as a function of the molar ratio ($[\text{heme_im}]/[\text{C}\beta\text{-casein}]$) (■) experimental, (—) fitted curve, $T = 27^\circ\text{C}$

Figure 12.

Progress curves for suicide inactivation of the catalyst: heme 3 μM + imidazole 3 mM+ SDS 0.9 mM+ C β -casein 3.25 μM with 1.5 mM hydrogen peroxide, as a function of guaiacol concentration over the reaction time. Data were fit to Eq. (5). Experimental (---) calculated (●) curves.

MKVLILACRVALALAREKEEFKTAGEALESISSEESITHINKQKIEKFKIEEQQTETEQQDKIYTFPQPQSLVYSHTPIPYIPILPQNF
LPPLQPAVMVPFLQPKVMDVPKTKETIIPKRKEMPLQSPVVPFTESQSLTLTDLENLHLPLLLQSLMYQIPQVPVQTPMIPPQSL
SLSQFKVLPVPQQMVPYQRAMPVQAVLPFQEPVDPVVRGLHPVPQPLVPVIA Camel β -casein

MKVLILACLVALALARELEELNVPGEIVESLSSEESITRINKKIEKFQSEEQQQTDELQDKIHFAQTQSLVYFPFGPIPNLQNP
LTQTPVVVPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLLLQSWMHQPHQPLPPTVMFPPQ
SVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV Bovine β -casein

Scheme 1.

The amino-acid sequence of the camel and bovine β -caseins. The shaded amino acids are the signal peptides from camel and bovine's milk β -casein.

Scheme 2.

The catalytic reaction cycle and inactivation pathway for peroxidase-like enzymes. figure^(Gharibi et al. 2011)

Table 1

Circular Dichroism analysis of secondary structures for different components.

C β -casein-heme	C β -casein-SDS-heme	C β -casein-SDS	C β -casein	Secondary structure
18.46	20.86	24.98	18.39	α -Helix
25.59	21.33	17.63	23.29	anti-parallel Beta sheet
9.87	9.87	9.40	10.15	Parallel beta sheet
19.01	18.70	18.61	18.57	Beta turn
27.15	29.23	29.38	29.62	Coil
≈ 100	100	100	≈ 100	Total

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Table 2
Calorimetric parameters for mixed β -casein-SDS-hemin-imidazole complexes at 27°C

Binding statues	N	K_b (M ⁻¹)	H (cal/mol)	S (cal/mol deg)
First binding set	0.69 ± 0.04	$\pm 4.42 \times 10^6 \times 10^7$	1.14 $-9.30 \times 10^5 \pm 8.51 \times 10^4$	-3.07×10^3
Second binding set	3.92 ± 0.36	$\pm 3.93 \times 10^3 \times 10^5$	2.12 $-4.54 \times 10^5 \pm 6.09 \times 10^4$	-1.49×10^3

Table 3

Kinetic parameters for the designed biocatalysts, the heme based biocatalyst concentrations were 12 μM (heme concentration) and HRP concentration was 3 nM

Biocatalyst	k_{cat} (s^{-1})	K_m (μM)	Catalytic efficiency ($\text{mM}^{-1}\text{s}^{-1}$)	Activity ($\mu\text{M}\cdot\text{min}^{-1}$)		Specific Activity ($\mu\text{Mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)
				[H ₂ O ₂]=1.5 mM,	[Guaiaacol]=0.22 mM	
Heme +C β -Casein +SDS	0.037	19.89	1.86	20.87	0.0026	
Heme+Imidazole+SDS	0.074	34.11	2.16	36.10	0.0046	
Heme+Imidazole [#]	0.077 [#]	32.06 [#]	2.39 [#]	37.81	0.0048	
Heme +C β -Casein+SDS+Imidazol (Caseoperoxidase)	0.12	6.66	17.66	69.73	0.0089	
Native HRP [*]	420 [*]	5800 [*]	72.4	14.25	0.108	

^{*} Ref (Moosavi-Movahedi et al. 2008),

[#]Ref (Gharibi et al. 2011)

Table 4

k_i and α_0 suicide values for the designed catalysts obtained during a time course of 11.2 min, by fittings to the progress curves.

Biocatalyst	[H ₂ O ₂] mM	k_i (min ⁻¹)	α_0 (min ⁻¹)
heme 3 μ M+ imidazole 3 mM+SDS 0.9 mM+ C β -casein 3.25 μ M	1.5	0.088	0.027
heme 3 μ M+ imidazole 3 mM+SDS 0.9 mM	1.5	0.103	0.028