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Esterase activity of bovine serum albumin up to 160 °C: A new benchmark for biocatalysis

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Abstract

Albumins are highly conserved proteins that carry out a multitude of physiological functions and exhibit a broad range of catalytic activities. Moreover, amino acid sequence comparisons of the albumin multigene family indicate that albumins diverged from a common ancestor. Here we report that bovine serum albumin (BSA) can catalyze ester hydrolysis at high temperatures (as high as 160 °C) well beyond the temperature limits reported for enzymatic catalysis, including for enzymes from known hyperthermophiles. Furthermore, BSA exhibited a ∼133-fold increase in its turnover number (k_{cat}) toward *p*-nitrophenyl palmitate from 70 to 150 °C. When BSA was incubated for 1 h at 150 °C in the presence of 25 mM SDS, it retained complete esterase activity, indicating that a catalytically competent orientation of amino acid residues exists in the denatured or partially unfolded protein. However, esterase activity diminished to ∼50% upon disruption of the protein's disulfide bridges and disappeared completely when BSA was digested by proteases. These results point to a new standard of robustness for biocatalytic activity at high temperatures. Catalytic activity and promiscuity at very high temperatures could have been advantageous to enzymes in primitive organisms evolving in hot environments, making BSA an intriguing model for early enzymes.

Keywords: Promiscuity; Ester hydrolysis; Hyperthermophilic enzyme activity; Thermal stability; Detergent effects

1. Introduction

Specificity is often considered to be a distinctive feature of biocatalytic activity. In recent years, however, it has become clear that some enzymes have additional functions outside of their primary catalytic reactivities. Some enzymes 'moonlight', serving non-catalytic regulatory functions, while others are catalytically promiscuous, having the ability to catalyze secondary reactions on multiple substrates within the same active site [1,2]. From an evolutionary perspective, such promiscuous activity (inefficient and rudimentary as it may be) could have provided ancestral cells with an adaptive advantage, enabling survival and providing a foundation for further evolution [3]. Furthermore, the relatively small number of enzyme superfamilies suggests that evolution has solved myriad cellular requirements with limited enzymatic resources [4,3]. These observations provide strong support for the central role of divergent evolution in biology, whereby catalytic promiscuity may be considered as a starting point for enzyme evolution [5].

Serum albumin is the most abundant protein in the sera of vertebrates and has been one of the most extensively studied of all proteins [6]. The primary structures of several species of albumin are known, and mammalian types show amino acid sequence identities of about 70–80%. In particular, human serum albumin (HSA) and bovine serum albumin (BSA) share a sequence homology of 76% [7]. In addition to its regulatory role of maintaining blood osmolarity, serum albumin also serves as a transport protein for fatty acids and can bind a diverse range of metabolites and xenobiotics [6,8,9]. In HSA, for example, five principal binding sites for medium- or long-chain fatty acids have been located [8].

A remarkable functional property of albumins is their promiscuous catalytic activity toward a broad range of organic molecules, including esters, amides, phosphates and benzisoxazoles [10–12]. In particular, the hydrolysis of *p*-nitrophenol esters by HSA was first reported in 1951 [13]. This "esterase-like" activity has been localized to the sub-domain IIIA of HSA [14,15]. HSA is characterized by a heart shape and is

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composed of three structurally similar domains (designated I–III), each of which is stabilized by an unusually large number of internal disulfide bonds (17 in total). Each domain consists of two sub-domains (A and B) that share common structural elements [9,8]. Site-directed mutagenesis studies have shown that Arg-410 and Tyr-411 are essential for the esterase activity of HSA, and a reaction mechanism similar to that of proteases has been proposed [15,16]. A covalent intermediate is formed in the active site, and throughout the catalytic reaction, Arg-410 remains hydrogen-bonded to Asn-391, suggesting the presence of a catalytic triad [17].

In the present work, we report ester hydrolysis by BSA at extraordinarily high temperatures beyond those previously reported for biological reactions. When considered in connection with the catalytic promiscuity of albumins, this extreme reactivity points to a model whereby high thermostability and promiscuous activity may have been synergistic properties of ancestral enzymes in primitive organisms evolving in high-temperature environments.

2. Material and methods

2.1. Chemicals

All the reagents, substrates and BSA (fatty acid-free, fraction V) were purchased from Sigma (St. Louis, MO, USA).

2.2. Measurement of protein concentration

The protein concentration was measured using the Bradford Bio-Rad (Hercules, California) protein microassay [18].

2.3. Determination of esterase activity

The hydrolysis of *p*-nitrophenyl acyl esters catalyzed by BSA was followed spectrophotometrically by monitoring the formation of *p*-nitrophenol (pNP) at 410 nm (AVIV Model 14DS UV–vis spectrophotometer). Typical assays were performed as follows: 900 μl of 50 mM Pipes buffer pH 7, containing 1 M NaCl and 50 μl of 0.1 mM BSA, were mixed together in a 1 ml-cuvette and pre-heated in a water bath at the assay temperature. For reaction temperatures >90 °C, the mixtures were pre-heated to 90 °C before the addition of 50 μl of 3 mM *p*-nitrophenyl acyl ester dissolved in isopropanol. This reaction mixture was immediately placed in the spectrophotometer set at the assay temperature. The background substrate hydrolysis was accounted for with blanks containing buffer instead of BSA for all conditions tested. All experiments were performed in duplicate or in triplicate, and the values in the figures and tables represent the means ± the standard deviation. Extinction coefficients for pNP at 410 nm were determined for each assay temperature. The calculated coefficients increased linearly from $10.04 \times 10^3\ M^{-1}\ cm^{-1}$ at 60 °C to $11.01 \times 10^3\ M^{-1}\ cm^{-1}$ at 160 °C. One unit (U) was defined as the release of 1 μmol of *p*-nitrophenol per minute.

2.4. BSA specificity

The BSA specificity was determined by assaying the activity of BSA towards *p*-nitrophenyl (pNP-) acyl esters having different chain lengths (C2 = acetate, C3 = propionate, C4 = butyrate, C6 = caproate, C8 = caprylate, C10 = caprate, C12 = laurate, C14 = myristate and C16 = palmitate). Assays times were either 5 or 15 min depending on the substrate.

2.5. Effect of SDS on esterase activity

The effect of SDS on BSA esterase activity was studied using two concentrations of pNP-myristate (50 and 500 μM) at 95 °C with NaCl excluded from the buffer solution. The assay buffer contained varying amounts of SDS ranging from 0 to 25 mM for solutions containing 50 μM substrate, and 0 to 65 mM for solutions containing 500 μM substrate. Tweens (polyoxyethylene detergents) and Tritons were avoided because their solutions became cloudy at high temperatures and interfered with the esterase activity assay.

2.6. Effect of incubation temperature on esterase activity

The effect of temperature on BSA activity was studied using pNP-palmitate as a substrate. For kinetic studies, the BSA concentration was decreased as the incubation temperature increased in order to increase the time over which the initial rate could be measured (from 10 μM for 70 °C to 0.25 μM for 150 °C or 160 °C). At temperatures >95 °C, the reaction system was pressurized to 60 psi using argon to prevent boiling of the reaction mixture. After placing the cuvette in the spectrophotometer, the reaction mixture reached a set-point temperature of 160 °C in 4 min. Consequently, for all assays above 95 °C, the rate of substrate hydrolysis was measured after 5 min of incubation at the set-point temperature. Spontaneous substrate hydrolysis was not observed at temperatures less than 150 °C, and only a slight background hydrolysis rate was observed and subtracted from assay data at 160 °C. The contribution of BSA aggregation at 150 °C to the optical density at 410 nm was negligible (<1%) compared to that of pNP release.

2.7. Effect of the substrate concentration on esterase kinetics

The effect of the substrate concentration (pNP-palmitate) on BSA esterase activity was examined at 70 and 150 °C in the absence of SDS. Kinetic parameters were determined from Lineweaver-Burk plots.

2.8. BSA digestion

BSA (0.4 mM) was dissolved in 100 mM Tris–HCl, pH 8.2, containing 8 M urea. The BSA disulfide bonds were reduced by adding dithiothreitol to a final concentration of 7 mM and incubating at 60 °C for 60 min. Free sulfhydryl groups were then blocked by adding iodoacetamide to a final concentration of 15 mM followed by incubation at 25 °C for 15 min in the dark. The sample was diluted eightfold with 100 mM Tris–HCl, pH 8, containing 1.2 mM $CaCl_2$, giving a final urea concentration of 1 M, and the unfolded protein was then digested by adding proteases (trypsin, chymotrypsin, or subtilisin A) in a molar ratio to BSA of 1:50. The digest solutions were then incubated for 36 h at 37 °C. Residual activities were assayed for each digestion step at 95 and 150 °C. Controls lacked the addition of proteases, but were incubated for 36 h at 37 °C.

2.9. BSA stability experiments

BSA solutions (100 μM) containing either 25 mM or no SDS were incubated at 150 °C for 1 h. Following the incubation, protein samples were centrifuged at 20,800 × *g* for 10 min and the residual esterase activity of 50 μl of the supernatant was assayed at 95 °C using pNP-myristate as the substrate. NaCl was excluded from the buffer solution and SDS (3 mM) was added instead.

2.10. Hydrolysis of other esters

The hydrolysis of other esters was measured according to previously published methods. The experiments were performed at 90 °C and the substrates and methods used were the following: 4-methylumbelliferyl-butyrate and 4-methylumbelliferyl-oleate by fluorescence [19], Tween 20 and Tween 80 by spectrophotometry [20], palmitin, tripalmitin and triolein by a colorimetric assay [21], tricaprylin and tributirin by titrimetry [22].

3. Results

BSA has been shown to exhibit optimal esterase activity toward *p*-nitrophenyl esters at pH 9.5 [16,17]. However, we carried out assays at pH 7.0 because the ester substrates had greater