Serum Albumin-Catalyzed Trigger System by Using a Tandem Kemp Elimination/β-Elimination Reaction

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The serum albumins are the most abundant proteins in blood serum and act as transport proteins for chemically diverse molecules, including long-chain fatty acids, amino acids, and steroids.[1] The principal regions of ligand binding are located in hydrophobic cavities, which have been identified in some detail in recent crystal structures.[2,3] Human serum albumin (HSA) is also of pharmaceutical interest as a carrier for numerous drug molecules.[4] Binding to HSA controls the free, active concentration of a drug, providing a reservoir for a long duration of action.

In addition to its unique binding properties, several weak catalytic activities have been detected for bovine serum albumin (BSA) and HSA, such as esterase-like activity,[5] Kemp elimination catalysis,[6,7] decomposition of a Meisenheimer complex,[8] and, more recently, catalysis of *S*-nitrosothiol formation.[9] Except for this last activity, the physiological significance of these catalytic properties has not been demonstrated. This albumin polyreactivity seems to be correlated to the basic active-site features of apolar pockets that can target several classes of hydrophobic substrates and act as a primitive active sites, thus allowing these promiscuous activities to take place.[10] The IIA binding site of HSA consists of a hydrophobic pocket surrounding a reactive lysine.[1,2] This site has been shown to catalyze the so-called Kemp elimination reaction in which the substrate 5-nitrobenzisoxazole decomposes into a cyanophenol compound.[6,7] A lysine side chain, Lys222 in BSA and the homologous Lys199 in HSA,[11] is thought to act as the catalytic base and trigger the reaction by abstracting the acid proton of the isoxazole cycle of the substrate. This "model" reaction was used to study the relative contribution of the medium effect due to the protein hydrophobic sites and general base catalysis in various proteins, including catalytic antibodies.[11–14] However, the potential of the benzisoxazole system as a protecting group was only exploited once by the Kemp group.[15]

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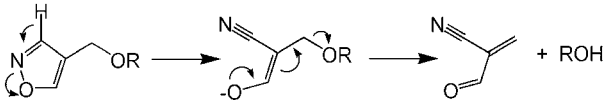
Here, we report the design of a new protective group that can be removed in vitro by using albumin as a natural catalyst. This approach is based on the catalysis of a reaction cascade that involves the opening of an isoxazole ring followed by a β-elimination reaction (Scheme 1). We show that this tandem reaction is efficiently catalyzed by BSA and HSA and could have a general applicability in a prodrug-activating strategy.[16]



Scheme 1. *Mechanism of the tandem Kemp elimination/β-elimination reaction (**1**: R = p-nitrophenoxy, **2**: R = o-nitrophenoxy, **3**: R = phenoxy, **4**: R = estrone)*

The cleavable group was designed from the isoxazole ring, which can be opened under basic conditions to give the corresponding derivative. If a methyl ether substituent is present at the 4-position in the isoxazole ring, this intermediate can subsequently undergo a β-elimination to give the alcohol product ROH and 3-cyanoacrolein. In water, this latter product, a strong Michael acceptor, is immediately hydrated. In order to test this approach, we synthesized the model substrates: 4-[(*p*-nitrophenoxy)methyl]isoxazole (**1**) and 4-[(*o*-nitrophenoxy)methyl]isoxazole (**2**), 4-(phenoxymethyl)isoxazole (**3**), which after the two coupled reactions should release phenol products.

The kinetics of these model substrates were first tested at different pH with and without BSA or HSA (Table 1). Without protein, these compounds exhibit a slow decomposition and their stability is related to the pK_a of the leaving group.[14] Both BSA or HSA were found to accelerate the rate of decomposition of these 4-(aryloxymethyl)isoxazoles, even at neutral pH. No time lag was observed in the time course of the release of phenols; this suggested that the secondary decomposition by β-elimination is not the rate-limiting step even over the pH range 7–10. This confirms that albumins are efficient catalysts for the β-elimination of β-(aryloxy)carbonyl compounds.[17] Multiple turnover was observed (see Supporting Information) before the rate gradually decreased due to nitrophenolate accumulation, which inhibits the albumin-catalyzed reaction above 60 μM (data not shown). The low solubility of the substrate (precipitation occurs above 300 μM) prevented measurement of the kinetic parameters, k_{cat} and K_m, of these albumin-catalyzed reactions, but as the initial velocities were proportional to substrate concentrations below 200 μM, we conclude that the K_m is higher than this value for both albumins. Assuming K_m values in the 1–5 mM range, which is reasonable for serum albumin according to the results with benzisoxazole,[6] k_{cat} values for BSA and HSA lie in the range $0.2–1\times10^{-3}\ s^{-1}$ for 4-[(*p*-nitrophenoxy)methyl]isoxazole at pH 8. Interestingly, the catalytic proficiencies ($k_{cat}/K_m/k_{uncat}$)[18] of BSA and HSA stay almost constant over the 8–11 pH range, as the high background rate at high pH is balanced by a higher efficiency of albumin catalysts. Indeed, as shown in Figure 1, the rate of the albumin-catalyzed reaction increased with pH. Analysis of this pH dependence suggests the participation, in its basic form, of

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Table 1. *Rate constants for the uncatalyzed and albumin-catalyzed decomposition of 4-(aryloxymethyl)isoxazoles.*

Leaving group	pK_a	pH	k_{uncat}[a] [s^{-1}]	BSA k_{cat}/K_m[b] [$M^{-1}s^{-1}$]	BSA $k_{cat}/K_m/k_{uncat}$ [M^{-1}]	HSA k_{cat}/K_m[b] [$M^{-1}s^{-1}$]	HSA $k_{cat}/K_m/k_{uncat}$ [M^{-1}]
4-nitrophenol	7.1	8	6.00×10^{-7}	0.2	3.3×10^{5}	0.22	3.7×10^{5}
		10	1.77×10^{-5}	0.92	5.2×10^{4}	1.31	7.4×10^{4}
		11	2.45×10^{-5}	2.73	1.1×10^{5}	2.35	9.6×10^{4}
2-nitrophenol	7.2	8	1.03×10^{-6}	0.0425	4.1×10^{4}	0.0202	1.9×10^{4}
		10	1.20×10^{-5}	0.17	1.4×10^{4}	n.d.	
		11	2.02×10^{-5}	0.36	1.8×10^{4}	0.57	2.8×10^{4}
phenol	9.9	8	2.33×10^{-8}	0.00667	2.9×10^{5}	n.d.	
		10	2.62×10^{-6}	0.00833	3.2×10^{3}	n.d.	

[a] Pseudo-first-order constants determined at 25 °C in phosphate buffer (30 mM) containing NaCl (100 mM) at pH 8, sodium carbonate buffer (30 mM) containing NaCl (100 mM) at pH 10 and 11. The rate of decomposition was followed by monitoring the absorbance at 405 nm for the nitro compounds and by HPLC for the phenol compound. [b] The reactions with HSA (25 μM) and BSA (25 μM) were performed in the same buffers at three different substrate concentrations (50 μM, 100 μM, and 200 μM).

the catalyzed reaction (Figure 3) with both albumins. Possible catalysis by the IIIa site is not excluded since this site contains a potentially basic residue, such as the Tyr411, which has been identified as the residue responsible for the low esterase activity of albumin.[20] Taken together, these data suggest a major contribution of the IIa site to the catalysis and that these coupled reactions are catalyzed by the same site as the one involved in the catalysis of the Kemp elimination by albumin. However, the IIa site of HSA seems to contribute to a greater extent to the catalysis of this tandem reaction since almost complete inhibition is observed with PyrP although only 40% inhibition

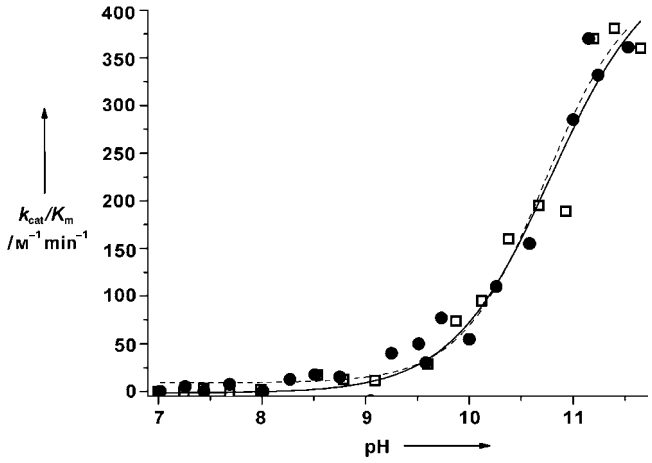


Figure 1. *Plot of k_{cat}/K_m versus pH for the decomposition of 4-[(p-nitrophenoxy)-methyl]isoxazole catalyzed by BSA (●) and HSA (□). Data were fitted to the equation $k_{cat}/K_m = (k_{cat}/K_m)_{max}/(1+10^{pK_a-pH})$, giving for BSA $pK_a = 10.7 \pm 0.1$ and $(k_{cat}/K_m)_{max} = 4.3\times10^2\ M^{-1}\ min^{-1}$, and for HSA $pK_a = 10.7 \pm 0.2$ and $(k_{cat}/K_m)_{max} = 4.5\times10^2\ M^{-1}\ min^{-1}$.*

an active-site residue with an apparent p$K_a \approx 10.7$ for both BSA and HSA. This also suggests that a general base is involved in the catalysis, which could correspond to the lysine group previously identified in the binding site of both albumins.

To further identify the albumin site where this reaction occurs and confirm the involvement of lysine as the active residue of the IIa site of BSA (Lys222) and HSA (Lys199), we used pyridoxal phosphate for specific side-chain modification. Pyridoxal phosphate forms a covalent adduct with BSA, the aldehyde group reacting with lysine side chains at two distinct sites.[19] As shown in Figure 2, up to 70% of the catalytic activity of BSA is inhibited by incubation with an excess of pyridoxal phosphate. HSA is almost completely inhibited under the same conditions. The observed residual activity with BSA suggests that other binding sites could be involved in catalysis as previously described for Kemp elimination.[11] Using an anionic ligand like octanoate, which is known to bind at several sites but preferentially at IIIa,[1] we observed complete inhibition of

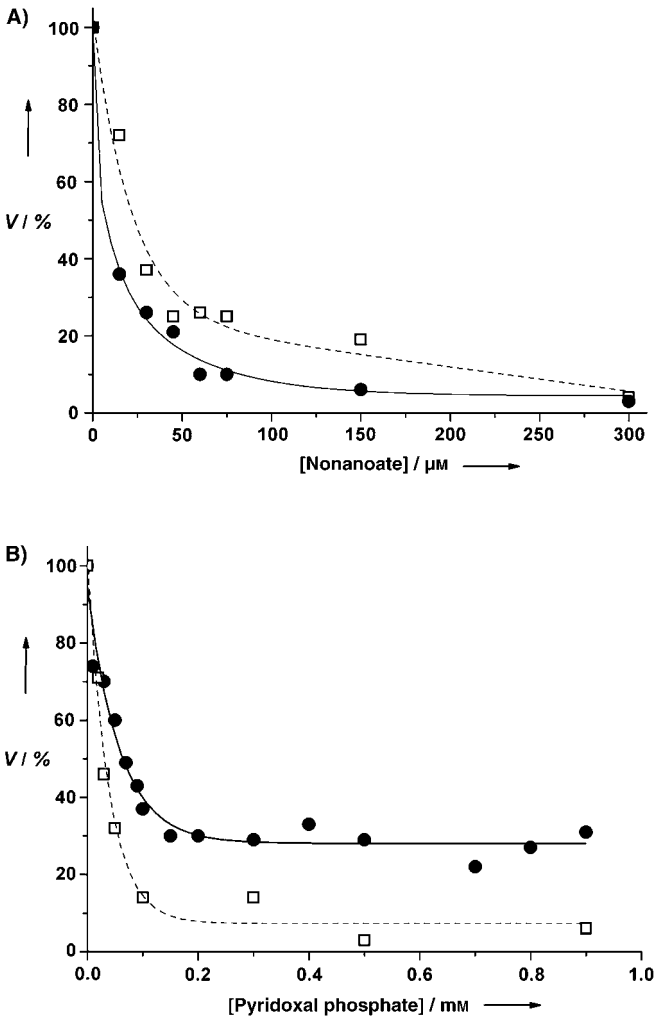


Figure 2. *Inhibition of BSA (●) and HSA (□) catalysis by A) nonanoate and B) pyridoxal phosphate. Residual activities (V) were determined with 4-[(p-nitrophenoxy)methyl]isoxazole and 15 μM of serum albumin at pH 9 (30 mM bicine, 100 mM NaCl).*

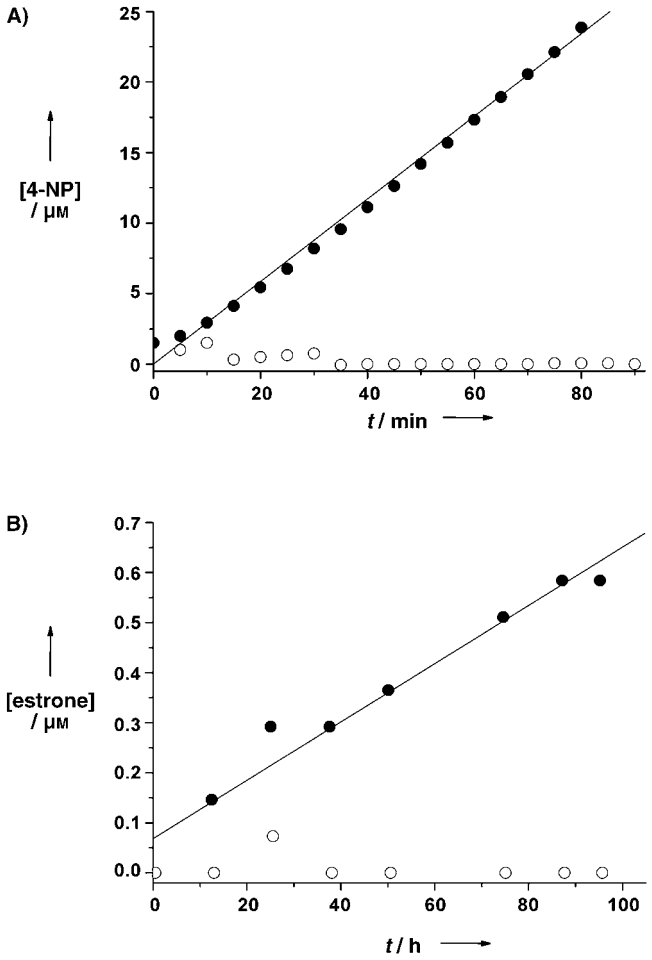
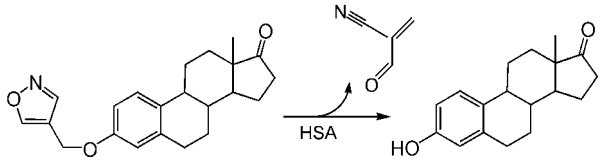


Figure 3. *A) Catalysis of the decomposition of 4-[(*p*-nitrophenoxy)methyl]isoxazole (**1**) by HSA. Substrate (250 μM) was added to the serum (pH 7.7) in the absence (●) or in the presence (○) of nonanoate (6 mM). The rate of decomposition was followed by monitoring the appearance of the* p*-nitrophenol product (4-NP) at 405 nm. B) Catalysis of the unmasking of estrone from prodrug **4** (25 μM) in the absence (○) or in the presence (●) of HSA (65 μM) at pH 7.4.*

was observed for the Kemp elimination.[11] One possible explanation for this discrepancy is that the narrow binding pocket IIa in HSA could be more accessible for substrate **1**, which presents a greater flexibility than the rigid nitrobenzisoxasole.

The applicability of this albumin-catalyzed tandem ring-opening/β-elimination reaction in the development of a new class of drug-modifying linkers and a prodrug-activating strategy is dependent on the in vivo stability of substrate **1** and on the efficiency of the product release at physiological pH. To test this concept, we measured the kinetics of the 4-nitrophenol release directly in human serum with or without an excess of nonanoate (Figure 3 A). In the presence of nonanoate, no release is observed over more than one hour; this demonstrates that the background reaction is very low and that no protein other than albumin is able to catalyze these tandem reactions. In the absence of nonanoate, a continuous release of the product was observed, even at a pH (7.7) far from the optimum value. This detectable activity is due to the naturally high concentration of albumin in serum (⩾67 μM), which compensates for the low efficiency of the catalyzed reaction at this pH. Moreover, to validate that this deprotection strategy is effective for specific serum prodrug activation, this masking chemistry was applied to the hormone estrone (Scheme 2). As shown



Scheme 2. *Activation of estrone prodrug **4** by a tandem Kemp elimination/ β-elimination reaction catalyzed by HSA.*

in Figure 3 B, a time-dependent release of the free estrone is observed in the presence of a physiological concentration of HSA (65 μM), whereas no nonspecific activation of the prodrug is observed in buffer solution at pH 7.4 without albumin over a period of three days. The catalytic efficiency, $k_{cat}/K_m = 10^{-3}\,M^{-1}s^{-1}$, is similar to that of the model compound 4-(methoxyphenyl)isoxazole; this indicates that the bulky steroid structure does not prevent accessibility to the albumin active site.

Thus, serum albumins are able to catalyze specifically the tandem ring-opening/β-elimination reactions that lead to deprotection of the model substrates as well as a real drug, providing that the drug contains a phenolic group. This system, which used a natural endogeneous catalyst of the blood, could be exploited to modulate drug pharmacokinetics in serum.[21] Finally, this strategy also appears to be a unique and specific way of detecting and measuring albumins in biological fluids by a chromogenic or fluorogenic reaction.

Experimental Section

Materials: All chemicals were reagent grade or better. Human serum albumin (fraction V) was from Calbiochem and bovine serum albumin (fraction V) was from Sigma (St Louis, MO; A-2153). Human serum was obtained from the Etablissement Français du Sang (EFS, Nantes). The volunteer donors agreed to give samples for research purposes.

Synthesis of 4-[(*p*-nitrophenoxy)methyl]isoxazole (1): A solution of 4-bromomethylisoxazole (for preparation, see ref. [22]) in dry DMF (2.5 mL) was added dropwise to a solution of *p*-nitrophenol (0.430 g, 3.1 mmol) and potassium carbonate (0.210 g, 1.54 mmol) in dry DMF (2.5 mL). The mixture was stirred for 2 h at RT. Then, the solution was diluted with water (10 mL) and extracted with CH_2Cl_2. The combined organic extracts were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification by flash chromatography (CH_2Cl_2) gave 4-[(*p*-nitrophenoxy)methyl]-isoxazole (80 mg, 30%). ^{1}H NMR (300 MHz, $CDCl_3$, 20 °C, TMS): $\delta =$ 5.09 (s, 2H; -CH_2-); 7.02 (d, $J = 9.2$, 2H; $HArNO_2$); 8.24 (d, $J = 9.2$, 2H; $HArNO_2$); 8.41 (s, 1H; -NCH-); 8.57 (s, 1H; -OCH-); ^{13}C NMR (75 MHz): $\delta = 59.84$, 114.62, 126.04, 142.11, 149.16, 156.32, 162.78, 171.32; MS (EI): *m/z* (%): 220 (4) $[M]^+$, 139 (43), 109 (14).

Synthesis of 4-[(*o*-nitrophenoxy)methyl]isoxazole (2): A solution of 4-bromomethylisoxazole (0.1 g, 0.6 mmol) in acetonitrile (2.5 mL) was added to a solution of *o*-nitrophenol (0.140 g, 1 mmol) and silver oxide (0.325 g, 1.2 mmol) in acetonitrile (2.5 mL). The mixture was stirred for 24 h at RT. Then, the solution was filtered and concentrated under reduced pressure. Purification by flash chromatography (CH_2Cl_2) gave 4-[(*o*-nitrophenoxy)methyl]-isoxazole (100 mg, 73%). ^{1}H NMR (300 MHz): $\delta = 4.99$ (s, 2H; -CH_2-), 7.00 (dd, $J = 7.8$, 8.5 Hz, 1H; $HArNO_2$), 7.18 (d, $J = 8.5$ Hz, 1H; $HArNO_2$), 7.59 (dd, $J = 7.7$, 7.8 Hz, 1H; $HArNO_2$), 8.12 (d, $J = 7.7$ Hz, 1H; $HArNO_2$), 8.36 (s, 1H; -NCH-), 8.49 (s, 1H; -OCH-).

Synthesis of 4-(phenoxymethyl)isoxazole (3): A solution of 4-bromomethylisoxazole in acetonitrile (2.5 mL) was added dropwise to a solution of phenol (70 mg, 0.75 mmol) and silver oxide (0.348 g, 1.5 mmol) in acetonitrile (2.5 mL). The mixture was stirred for 24 h at RT. Then, the solution was filtered and concentrated under reduced pressure. Purification by flash chromatography (CH_2Cl_2) gave 4-(phenoxymethyl)isoxazole (15 mg, 12%). ^{1}H NMR (300 MHz): $\delta =$ 4.98 (s, 2H; -CH_2-), 6.95 (d, $J = 7.8$ Hz, 2H; HAr), 7.01 (t, $J = 7.5$ Hz, 1H; HAr), 7.33 (t, $J = 7.8$ Hz, 2H; HAr), 8.37 (s, 1H; -NCH-), 8.49 (s, 1H; -OCH-).

Synthesis of 4-[(3-hydroestra-1,3,5(10)-triene-17-one)methyl]-isoxazole (4): Dry DMF was added dropwise to a suspension of estrone (0.65 g, 2.4 mmol) in dry acetonitrile (5 mL) until the estrone was fully dissolved. Then silver oxide (1.10 g, 4.8 mmol) and a solution of 4-bromomethylisoxazole in acetonitrile (2.5 mL) were added. The mixture was stirred for 48 h at RT, then was filtered, concentrated under reduced pressure, and diluted with CH_2Cl_2 (10 mL). The solution was then washed with water (5×10 mL), and the combined organic extracts were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification by flash chromatography (CH_2Cl_2/MeOH 99.5:0.5) gave **4** (62 mg, 7%). ^{1}H NMR (300 MHz): $\delta = 0.91$ (s, 3H; -CH_3), 1.43–1.63 (m, 6H), 1.95–2.55 (m, 7H), 2.87–2.90 (m, 2H), 4.96 (s, 2H; -CH_2-), 6.69 (s, 1H; HAr), 6.75 (d, $J = 8.7$ Hz, 1H; HAr), 7.22 (d, $J = 8.7$ Hz, 1H; HAr), 8.36 (s, 1H; -NCH-), 8.48 (s, 1H; -OCH-).

Kinetic measurements: Initial rates for substrates **1** and **2** were determined spectrophotometrically by monitoring the release of 4-nitrophenol at 405 nm for **1** and the release of 2-nitrophnenol at 414 nm for **2** with a microtiter plate reader (iEMS, Labsystem). The release of phenol from substrate **3** and of estrone from **4** were followed at 211 nm and at 220 nm, respectively, by reversed-phase HPLC (Jasco PU-2089, UV-2075, AS-2057) with a C-4 column (Sephasil protein 250/4.6, 5 μ) by using various proportions of methanol/water at 0.75 mL min^{-1}. Initial velocities were determined at 25 °C with substrates over the 25–200 μM range and serum albumins (15–65 μM), and were corrected for the rate of the background reaction under the same conditions. Methanolic stock solutions of substrates were added to a solution of serum albumin in buffer (30 mM, NaCl 100 mM): sodium phosphate ($6 < pH < 8$), bicine ($8 < pH < 9$) or sodium carbonate ($pH > 9$), so that the final methanol concentration did not exceed 20% of the final volume. Apparent second-order rate order constants (k_{cat}/K_m) were obtained directly from the slopes of initial velocities against substrate concentrations at low substrate concentration (< 200 μM). For noncovalent inhibition, serum albumins were incubated with nonanoate (0–3 mM) for 30 min before measuring initial rates for the 4-[(*p*-nitrophenoxy)methyl]isoxazole as described before. Covalent inhibition was determined from residual initial rates after incubation of serum albumin with pyridoxal phosphate (0–0.9 mM) for 10 h in the dark.

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