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Human serum albumin as a catalyst of RNA cleavage: N-Homocysteinylation and N-phosphorylation by oligonucleotide affinity reagent alter the reactivity of the protein

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ABSTRACT

Kinetic parameters for the cleavage of UpA site in an oligonucleotide in the presence of human serum albumin (HSA) or one of its clinically relevant modification were measured. The RNA-hydrolyzing activity of HSA was decreased by its nonenzymatic N-homocysteinylation. According to ^{31}P NMR data, Lys and Tyr residues were the labeling targets when a phosphorylating analog of oligoribonucleotide substrate was employed. The site of tyrosine modification was slowly dephosphorylated. Lys-directed affinity labeling suppressed oligonucleotide cleavage indicating that lysines took part in the reaction.

Serum albumin (SA) is the most abundant protein in the sera of vertebrates and has been one of the most extensively studied of all blood proteins.[1] Studies of SA have revealed the important role of this protein as a catalyst for the hydrolysis of various compounds, such as esters, amides and organophosphorous compounds.[2] Moreover, the catalytic activity of BSA toward RNA molecules has been demonstrated.[3]

Recently we have reported in detail the RNA-hydrolyzing property of albumin from human serum (HSA).[4] In particular, it has been shown that glycation, which is one of the clinically relevant modifications of the protein in human blood, decreases the catalytic activity of HSA toward synthetic oligoribonucleotides. However, the albumin fraction in blood contains other modified protein forms, one of which is non-enzymatically N-homocysteinylated albumin (Hcy-HSA). It accounts for 0.26–0.36% of albumin in circulation in norm and increases in pathology.[5] Major pathophysiological consequences of protein N-homocysteinylation include induction of anti-*N*-Hcy-protein auto-antibodies and thrombogenesis, which contribute to atherosclerosis and cardiovascular diseases in human.[6] The main targets of N-homocysteinylation are known to be Lys-525, Lys-4, and Lys-12 albumin residues.[7] Since glycation, which is also lysine-directed,[8] decreases RNase-like activity of albumin,[4] N-homocysteinylation might also affect the catalytic activity of HSA.

In the current study, the RNA-hydrolyzing activity of albumin and its clinically relevant modification—N-homocysteinylated protein—has been examined in detail. In addition, HSA affinity labeling has been employed to reveal the type of amino acid residues in the protein active site.

A sample of nonenzymatic N-homocysteinylated albumin was prepared in vitro under the conditions described earlier.[9] According to MALDI-TOF MS, the obtained Hcy-HSA contained two homocysteine residues per protein molecule. Moreover, the formation of two additional thio-groups as a result of albumin modification was demonstrated in Ellman's assay.

To monitor the reaction of native and N-homocysteinylated albumin with the oligoribonucleotides, we employed the method of denaturing PAGE analysis using 5′-^{32}P-labeled oligonucleotides pAGGAUCUAUAAAUGAC (ON16) or pd(AGGATC)rUd(ATAAATGAC) (dON16) as substrates. For the analysis of protein-mediated cleavage the substrates were incubated with HSA or Hcy-HSA for definite time at 37 °C in a solution containing 50 mM Tris-HCl, pH 7.0, 0.2 M KCl, and 0.5 mM EDTA. The reaction was quenched by precipitation of the oligonucleotide cleavage products with 6 % $LiClO_4$ in acetone, followed by 20% PAGE/7 M urea analysis. Quantitative data were obtained by digitizing the autoradiograph of the gel using GelPro Analyzer software (Media Cybernetics). The degree of cleavage (%) was determined as a ratio of the radioactivity of the cleavage products to the total radioactivity of the sample applied onto the gel.

The PAGE revealed that Hcy-HSA, as well as HSA, promoted ON16 cleavage in a concentration-dependent manner (data not

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shown). For both HSA and Hcy-HSA the major cleavage occurs within the U7–A8 and U9–A10 sequences (Fig. 1A). These data are in accordance with the cleavage specificity of glycated albumin (gHSA).[4] Equivalent amounts of N-homocysteinylated albumin had smaller effect on ON16 hydrolysis (Fig. 1A). For instance, after 5 h of incubation with native HSA (1×10^{-6} M) about 30% of ON16 (1×10^{-5} M) was cleaved, whereas Hcy-HSA induced only ~2% cleavage under the same conditions. This result suggested that nonenzymatic N-homocysteinylation of HSA decreased the RNase-like activity of the protein.

Since the major ON16 cleavage site was UpA, for determining the kinetic parameters of albumin-mediated RNA hydrolysis we used single-stranded oligodeoxyribonucleotide dON16 containing a ribonucleotide forming a cleavable phosphodiester bond between U7 and A8 residues.

The reaction of the dON16 with native or modified HSA was characterized by saturation kinetics typical for enzyme-catalyzed reactions (Fig. 1B). However, the catalytic parameters of albumin-mediated dON16 hydrolysis were determined using a formalism suggested earlier[2a] rather than the standard Michaelis–Menten equation because in our case the initial concentration of the proteins was of the same order as that of the substrate. For the calculations velocity data were obtained from the initial rate values. Data from Figure 1B were fitted to Eq. 1 to provide the values for the turnover number (k_{cat}) and the dissociation constants (K_s) for the complexes of HSA and Hcy-HSA with dON16 (Table 1).

$$v = \frac{k_{cat}}{2} \times \{([S]_0 + [E]_0 + K_s) - \sqrt{([S]_0 + [E]_0 + K_s)^2 - 4([E]_0[S]_0)} \quad (1)$$

According to the available data for RNase A, the rate constants for the hydrolysis of UpA dinucleotide is 3.5×10^3 s^{-1}.[10] As can be seen in Table 1, the rate of albumin-mediated hydrolysis of phosphodiester bond in UpA site is much slower. HSA catalyzes the hydrolysis of dON16 at 37 °C with the catalytic efficiency (k_{cat}/K_s) of 1.9×10^2 M^{-1} s^{-1}. For the cleavage of phosphodiester bond in UpA dinucleotide by RNase A, this value is 1.7×10^6 M^{-1} s^{-1}.[10] Thus, the HSA-catalyzed cleavage of UpA is four orders of magnitude slower than that by RNase A. Though the RNase-like activity of HSA is low, albumin may play a role in the hydrolysis of extracellular RNA due to its high concentration in human plasma (~0.6 mM). N-Homocysteinylation of protein resulted in significant loss of its RNA-hydrolyzing activity (the ratio $k_{HSA}/k_{Hcy\text{-}HSA} = 20$).

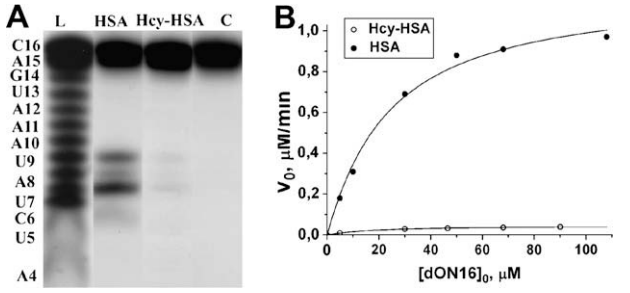


Figure 1. (A) Autoradiograph of 20% PAGE/7 M urea after analyzing the products of ON16 cleavage in the presence of native or N-homocysteinylated HSA. Lane L represents the imidazole ladder; lane C, incubation of ON16 without proteins. Cleavage conditions: 50 mM Tris–HCl, pH 7.0, 0.2 M KCl, 0.5 mM EDTA, 1×10^{-5} M [α-^{32}P]ON16, 1×10^{-6} M HSA or Hcy-HSA, 37 °C, 5 h. (B) The kinetic parameters for dON16 hydrolysis in the presence of HSA or Hcy-HSA. Initial rates of product release ($v0$) were determined at a range of substrate concentrations ([S]$_0$ = 5–100 μM; [HSA] = 5 μM). Data were fitted to Eq. 1.

Table 1
Kinetic parameters for dON16 cleavage in the presence of HSA and Hcy-HSA

Protein	K_s (μM)	k_{cat} (min^{-1})	k_{cat}/K_s (M^{-1} s^{-1})
HSA	21 (±3)	0.24 (±0.01)	1.9×10^2
Hcy-HSA	16 (±2)	0.0091 (±0.0002)	9.5

There is an analogy between glycation and N-homocysteinylation of HSA. Earlier,[11] we have reported that the affinity of glycated HSA toward polyadenylic acid is almost twice as high as that of the unmodified protein. Here, we demonstrate that N-homocysteinylated albumin binds dON16 slightly more efficiently than native protein (Table 1). N-Homocysteinylated and glycated albumins are formed in vivo by nonenzymatic reaction between ε-amino groups of exposed lysine side chains within the protein and Hcy-thiolactone and D-glucose, respectively.[7,8] Modification of lysines suppressed the hydrolysis of oligoribonucleotides, leading to the conclusion that Lys residues take part in the reaction. Additional support for this conclusion comes from our studies on chemical cross-linking of HSA with an oligonucleotide substrate analog.

Amidophosphates of oligoribonucleotides with zwitter-ionic terminal phosphate group containing 4-*N*,*N*-dimethylaminopyridine (DMAP) residue have been used to interrogate active sites and functional domains of proteins for critical lysine residues, exploiting mainly lysine-directed modification by these agents.[12] Therefore, we used DMAP derivative of p(U)$_6$ for HSA affinity studies. This compound reacts with proteins as a phosphorylating reagent and hence can in principle attack His, Arg, Tyr, Thr, Ser, Cys, Asp, and Glu residues. The nature of the formed oligonucleotide derivatives could be revealed by ^{31}P NMR due to the difference in chemical environment of the phosphorus atom.[13]

According to the ^{31}P NMR data (Fig. 2A) a 3 h reaction of albumin (10^{-3} M) with DMAP-p(U)$_6$ (**1**) (10^{-3} M) resulted in phosphorylation of lysine (**2**) and tyrosine (**3**) residues of the protein (Scheme 1).

In ^{31}P NMR spectrum of the reaction mixture peaks of the terminal 5′-phosphate derivatives (the −5.6 and 9.8 ppm resonance peaks in Figure 2A, panel a) were observed instead of a broader signal (4.3 ppm) for p(U)$_6$ (Fig. 2A, panel c). It is known that the downfield shifts of the ^{31}P NMR signals by 6–10 ppm, as compared to those of phosphomonoesters, are typical of aliphatic amidophosphates.[13] In this connection, the signal at 9.8 ppm (Fig. 2A, panels a and b) can be assigned to the amidophosphate derivative of lysine (**2**). The upfield shift of the terminal phosphate signal by ~6 ppm is characteristic for phenyl esters of nucleotide.[13] Therefore, the signal at −5.6 ppm in the spectrum (Fig. 2A, panel a) supported the structure (**3**) (Scheme 1), where the terminal phosphate group is bound to the phenolic oxygen of tyrosine.

After 23 h of incubation, phosphorylated Tyr released the bound oligonucleotide. At this point of the reaction, the ^{31}P NMR spectra showed disappearance of the resonance peak at −5.8 ppm with the concomitant appearance of resonance peak at 4.3 ppm (Fig. 2A, panel b). This peak can be assigned to the terminal phosphate group in p(U)$_6$ (Fig. 2A, panel c). Such a release can be described as demodification. The fact that after 23 h of reaction between albumin and DMAP-p(U)$_6$, the adduct peaks at 9.8 ppm have the same intensity indicates that the amidophosphate bond between lysine and p(U)$_6$ is stable.

The ^{31}P NMR spectra analysis indicated that the synthesis of DMAP-p(U)$_6$ resulted in the formation of small quantity of the products containing 2′,3′-cyclophosphate group (the 20.45 ppm peak in Fig. 2A). It is known,[14] that all methods of activation of unprotected oligoribonucleotide terminal phosphate groups are imperfect since they lead to isomerization and cleavage of internucleotide bonds. The reactive oligoribonucleotide derivatives carrying phosphorylating group (DMAP-p(U)$_6$) was prepared by the treatment of p(U)$_6$ in DMF with the mixture of triphenylphosphine and 2,2′-dipyridyldisulfide in the presence of 4-*N*,*N*-dimethylami-

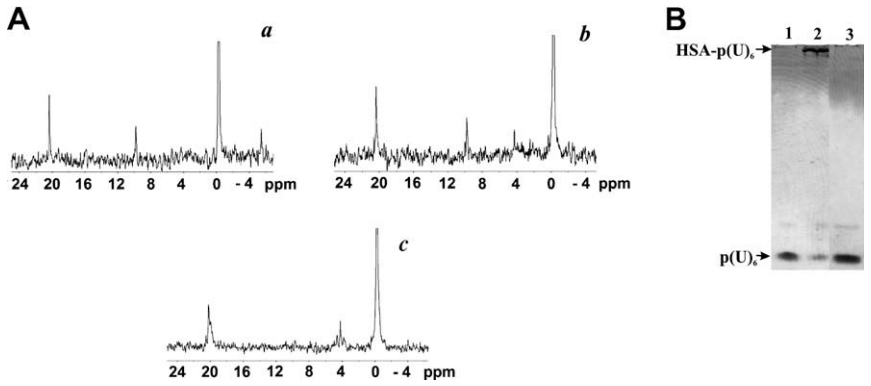
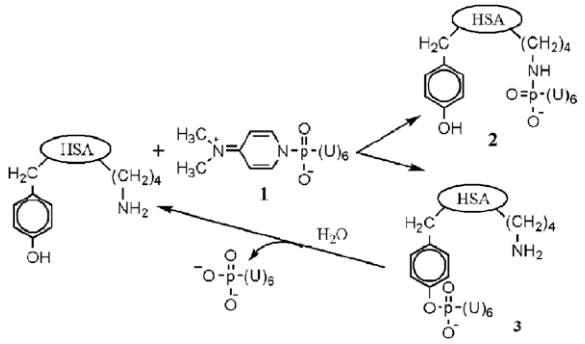


Figure 2. (A) ^{31}P NMR spectra of DMAP-p(U)6 (1×10^{-3} M) incubated in the presence of native HSA (1×10^{-3} M) in D2O at 27 °C for 3 h (a) and 23 h (b) and in the absence of HSA (c). (B) 20% PAGE/7 M Urea with subsequent stains-all staining. Lane 1, p(U)6; lane 2, HSA incubated with DMAP-p(U)6 for 23 h at 27 °C; lane 3, HSA preincubated with 10-M excess of p(U)6 followed by incubation with DMAP p(U)6.



Scheme 1. HSA modification with a phosphorylating oligonucleotide analog.

nopyridine according to the published procedure.[12] Triphenylphosphine and 2,2′-dipyridyldisulfide mixture was used as a reagent for synthesis of oligoribonucleotide derivatives (**1**) since it interacted with internucleotide phosphate groups in much smaller extent than other condensing reagents.[14]

We found that DMAP-p(U)$_6$ reacted with HSA stoichiometrically. According to MALDI-TOF MS, the phosphorylated albumin contained one oligonucleotide residue per protein molecule. Figure 2B clearly shows that oligonucleotide–albumin conjugate (lane 2) had lower electrophoretic mobility than the parent p(U)$_6$ (lane 1), which is consistent with considerable increase in the oligonucleotide molecular weight upon conjugation.

To confirm whether the labeling occurred in the oligonucleotide-binding site of HSA, the protective effect of the oligonucleotide excess was tested. For this purpose, HSA (10^{-3} M) was preincubated for 30 min with 10-fold molar excess of p(U)$_6$ in respect to the DMAP-p(U)$_6$, concentration. Then, standard concentration of DMAP-p(U)$_6$ (10^{-3} M) was added, and the incubation was continued for 23 h. Figure 2B clearly shows that HSA-p(U)$_6$ conjugate in lane 3 is undetectable, thus indicating that p(U)$_6$-protected albumin from cross-linking. In this connection, labeling of albumin by DMAP-p(U)$_6$ occurred inside the oligonucleotide-binding site of the protein due to the covalent attachment of the substrate analog to an amino acid residue of the enzyme contacting with the phosphate group. Moreover, when albumin was labeled with 1 mole of DMAP-p(U)$_6$, ~30% of the protein catalytic activity was lost, supporting the conclusion that the oligonucleotide derivative was attached to albumin in the vicinity of its active center.

Keeping in view the earlier studies[15] and going by the data of the present study, it can be concluded that such amino acid as lysine is important for the manifestation of the catalytic activity of RNA-hydrolyzing enzymes. Modification of lysine residues suppressed albumin-mediated hydrolysis of RNA, which may have an important physiological consequence. For example, the elevated level of lysine-directed HSA modifications may lead to the increase of the level of extracellular nucleic acids. Additional analysis should be made to elucidate the details of molecular mechanism for albumin-catalyzed RNA degradation and the role of the protein in the metabolism of nucleic acids.

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Supplementary data

The details of the experimental procedures can be found in Supplementary data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.09.049.

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