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Protein covalent modification by biologically active quinones^{*}

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Abstract: The avarone/avarol quinone/hydroquinone couple shows considerable antitumor activity. In this work, covalent modification of β -lactoglobulin by avarone and its derivatives as well as by the synthetic steroidal quinone 2,5(10)-estradiene-1,4,17-trione and its derivatives were studied. The techniques for studying chemical modification of β -lactoglobulin by quinones were: UV/Vis spectrophotometry, SDS PAGE and isoelectrofocusing. SDS PAGE results suggest that polymerization of the protein occurs. It could be seen that the protein of 18 kD gives the bands of 20 kD, 36 kD, 40 kD, 45 kD, 64 kD and 128 kD depending on modification agent. The shift of the pI of the protein (5.4) upon modification toward lower values (from pI 5.0 to 5.3) indicated that lysine amino groups are the principal site of the reaction of β -lactoglobulin with the quinones.

Keywords: quinone, avarone, steroidal quinones, β -lactoglobulin, covalent modification.

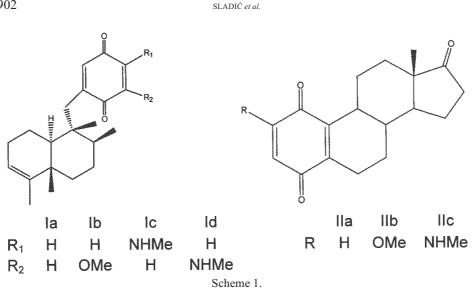
INTRODUCTION

The sesquiterpene quinone/hydroquinone couple avarone/avarol, isolated from the marine sponge *Dysidea avara*, and some of their derivatives exhibit a potent antitumor activity. Their activity can be attributed to generation of oxygen radicals leading to DNA damage,^{1–3} inhibition of enzymes² and microtubule assembly,⁴ as well as to alkylation of cellular nucleophiles by the quinone moiety.^{5,6} Avarone was found to modify sulfhydryl groups both in glutathione and in bovine serum albumin.⁵ In our previous publication on this subject, it was shown that avarone (**Ia**, Scheme 1) and some of its alkylthio derivatives covalently modify lysine amino

^{*} Dedicated to Professor Živorad Čeković on the occasion of his 70th birthday.

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groups in the model protein β -lactoglobulin (β -LG),⁷ and that the biological activity is partly correlated to ability of the quinone to modify the protein. Namely, those alkylthic derivatives that do not modify the protein also have the lowest antitumor activity.⁶ β-Lactoglobulin was selected as model protein because it has a relatively low molecular mass and several lysine residues,⁸ but only one, hindered, cysteine residue.⁹ Besides that, it is readily available and can be isolated in a very pure state. In order to confirm the generality of the reaction of β-lactoglobulin with quinones, in this work chemical modification of β-lactoglobulin by avarone derivatives with electron-donating substituents was studied. Such derivatives were chosen because they are less susceptible to nucleophilic attack. We also compared the protein modifying ability of compounds Ia-Id, which all have a rearranged drimane skeleton, with those of steroidal quinone 2,5(10)-estradiene-1,4,17-trione (IIa) and its derivatives (IIb,c).

EXPERIMENTAL

Avarol was isolated from fresh samples of D. avara, collected in the Bay of Kotor, and oxidized to avarone with silver oxide.¹⁰ The steriodal quinone 2,5(10)-estradiene-1,4,17-trione was prepared as presented earlier.¹¹ Methoxy and methylamino derivatives of the above mentioned sesquiterpene and steroidal quinones were synthesized as previously reported.¹²⁻¹⁴

β-Lactoglobulin, 18 kD protein, was isolated from cow milk by a standard procedure.¹⁵ The obtained protein solution was concentrated by ultrafiltration using Amicon P10 membrane and then lvophilized.

Modifications of β-lactoglobulin were performed in 20 % EtOH containing 50 mM NaHCO₃. The concentrations of the protein and the quinones were 5 mg/ml and 2 mg/ml, respectively. By optimization experiments, these concentrations were found to be the most suitable ones. The final volume was 1.2 ml, and the reaction was carried out at r.t. with stirring. After 48 h, the mixture was desalted on a PD-10 desalting column (Amersham Biosciences) by the standard procedure¹⁶ and the protein was eluted in the void volume.

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The chemical modification of β -lactoglobulin was followed by UV/VIS spectrophotometry (Beckman DU-50 spectrophotometer), SDS PAGE and isolectrofocusing.

SDS PAGE was performed under reducing conditions. The molecular masses of denatured protein samples were determined on 10 % polyacrylamide gel using molecular markers of the 20.0 kD, 29.0 kD, 43.0 kD, 67.0 kD and 97.4 kD (Pharmacia, Uppsala, Sweden).¹⁷ Isoelectrofocusing was performed on the 7.5 % polyacrylamide gel under nonreducing conditions with ampholite range from 3–10 and a calibration pI kit (Broad pI kit 3.5–9.3, Pharmacia, Uppsala, Sweden).¹⁸

The number of sulfhydryl groups per molecule of protein was determined using Ellman's reagent.¹⁹

RESULTS AND DISCUSSION

Confirmation that the reaction between the quinones and β -lactoglobulin takes place was obtained from UV/Vis spectra (Fig. 1). The modifications of the protein were visible instantaneously by the change of colour of the reaction mixture after mixing the quinone and the protein solutions. Spectral changes were visible for all tested compounds, the absorption maxima being shifted toward higher wavelengths (Fig. 1). The wavelengths of the absorption maxima are close to those of alkylamino derivatives of avarone, including alanino derivative, and the corresponding derivatives of steroidal quinones,^{12–14} so that it can be assumed that reaction with protein amino groups occurs.

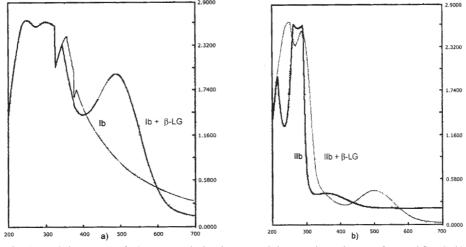


Fig. 1. UV/Vis spectra of: a) avarone derivative **Ib** and the reaction mixture of **Ib** and β -LG; b) steroidal derivative **IIb** and the reaction mixture of **IIb** and β -LG.

The potein modification was further investigated by SDS PAGE (Fig. 2). Avarone **Ia** and its derivative 3'-methoxyavarone **Ib** modified the protein of the 18 kD and gave strong bands of 36 kD, 64 kD and 128 kD. Steroidal quinone **IIa** also modified the protein and gave strong bands of 20 kD and 45 kD. Derivatives 4'-methylaminoavarone **Ic** and 3'-methylaminoavarone **Id** gave very sharp bands of 40 kD and 45 kD, and derivatives 2-methoxy-2,5(10)-estradiene-1,4,17-trione SLADIĆ et al.

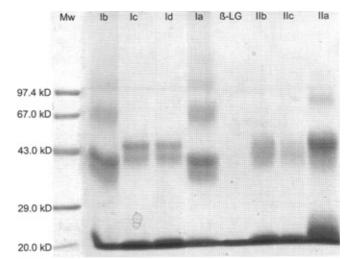
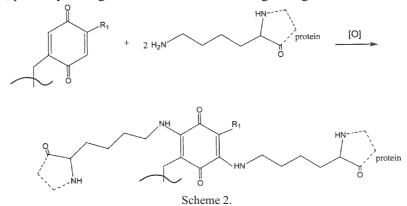


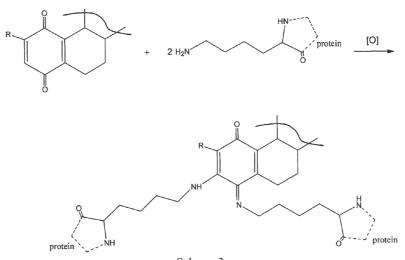
Fig. 2. SDS PAGE electrophoregram.

IIb and 2-methylamino-2,5(10)-estradiene-1,4,17-trione **IIc** gave a low-intensity band of 40 kD. These results suggest that polymerization of the protein occurs in all cases. The polymerization is most pronounced with avarone, less with its derivatives and the unsubsituted steroidal quinone, and the least with the substituted steroidal quinones. The results also indicate that various residues are the target of modification, since derivatives **Ic** and **Id** give two bands of relatively close apparent molecular masses, probably arising from difference in unfolding during denaturation.



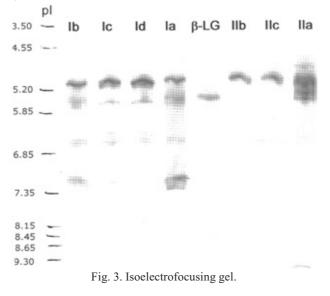
The protein contains four disulphide bonds and one cysteine residue which is not reactive because it is situated in the interior of the molecule.⁸ On the other hand, the protein contains 15 reactive ε -amino groups of lysine residues.⁹ Therefore, in this process the most likely reaction is the Michael addition of lysine NH₂ groups to the quinone nucleus (Scheme 2). Radical reactions seem unlikely in view of the fact that 3',4'-ethylenedithioavarone, a trisubstituted derivative with which the Michael addition does not occur, but which has a redox potential similar to

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Scheme 3.

those of protein-modifying monoalkylthio derivatives, does not modify the protein.⁷ Wtih steroidal quinones, since the polymerization occurs to some extent with the derivatives having only one position suitable for the Michael addition, quinone imines are likely to be involved (Scheme 3).



In order to ascertain whether the lysine ε -amino groups were the target of modification, the system was studied by nonreducing isoelectrofocusing (Fig. 3), which is often used as a method of choice for studying the nature of groups which are modified. The shift of the pI of the protein (pI = 5.4) upon modification toward lower values (5.0–5.3) indicated that the lysine amino groups of the protein are in-

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deed the principal sites of the reaction with the quinones. Because of nonreducing conditions of IEF, the polymers of high molecular weights can not completely enter the gel. The result is a characteristic smir of all protein bands except for modification with **IIb** and **IIc**, probably because these two give only one modificate of relatively low molecular weight. The confirmation that cysteine sulfhydryl group does not react with the quinones used was obtained by determination of mercapto groups¹⁹ before and after modification.

CONCLUSION

In conclusion, it was shown that avarone and its derivatives modify ε -amino groups of lysine residues in β -lactoglobulin. The steroidal quinones, especially the substituted ones, modify the protein to a lower extent. Therefore, it seems reasonable to assume that the sesquiterpene moiety in avarone enables proper positioning and non-covalent binding of the compound to the protein, which is the first, necessary step for the subsequent reaction. Influence of the sesquiterpene and steroid parts of the molecules, *i.e.*, the overall conformation, on the reaction with proteins might at least in part explain their specificity of action to particular types of tumor cells, ¹ although additional evidence is necessary. Still, the obtained results give new information on the mechanism of action of both avarone derivatives and steroidal quinones, indicating covalent modification of biomolecules as a possible mode of action.

These results might have additional significance in view of the fact that β -lactoglobulin was found to show antiviral activity upon modifications which change the charge density of the protein.²⁰

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ИЗВОД

КОВАЛЕНТНЕ МОДИФИКАЦИЈЕ ПРОТЕИНА БИОЛОШКИ АКТИВНИМ ХИНОНИМА

Душан сладић 1, ирена новаковић 2, зоран вујчић 1, татјана божић 1, наташа божић 2, драгана милић 1, богдан шолаја 1 и мирослав ј. гашић 1,2

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Хинонско/хидрохинонски пар аварон/аварол показује значајну антитуморску активност. У овом раду проучаване су ковалентне модификације β-лактоглобулина авароном, синтетичким стероидним хиноном 2,5(10)-естрадиен-1,4,17-трионом и њиховим дериватима. Технике за праћење хемијске модификације биле су: UV/Vis спектрофотометрија, SDS PAGE и изоелектрофокусирање. Резултати SDS PAGE указују да се дешава полимеризација протеина. Може се видети да протеин од 18 kD даје траке од 20 kD, 36 kD, 40 kD, 45 kD, 64 kD и 128 kD у зависности од агенса за модификацију. Померање рІ вредности протеина (5,4) након модификације ка нижим вредностима (од рІ 5,0

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до 5,3) показује да су амино-групе лизина главна места реакције β-лактоглобулина са хинонима.

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