# EFFECT OF ENZYMICALLY GENERATED ELECTRONIC-EXCITED SPECIES ON MEMBRANE-BOUND $Na^+,K^+$-ATPase

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The effect of electronic-excited species generated by systems 2-methylpropanal (MPAL) plus horse-radish peroxidase (HRP) and glyceraldehyde (GCA) plus HRP on $Na^+,K^+$-ATPase activity was investigated. For the system MPAL–HRP (which generates triplet acetone) $Na^+,K^+$-ATPase activity, the concentration of malondialdehyde, the membrane lateral pressure and the collisional quenching of protein tryptophan residues by NaI were determined. The enzyme activity decreased by 70% during the incubation. Lipid peroxidation products were increased to a steady state level from 5.5 ± 0.5 to 22.6 ± 0.7 nmol malondialdehyde $mg^{-1}$ protein after 60 min. The membrane lateral pressure was increased from 83.6 ± 0.4 to 90 ± 0.9 mN $m^{-1}$. In peroxidized membranes, the fluorescence intensity of tryptophan residues was decreased and the fluorescence intensity of bityrosine adducts was increased. Changes of conformation of $Na^+,K^+$-ATPase were observed by the collisional quenching of protein tryptophan residues by NaI. For the system GCA–HRP the $Na^+,K^+$-ATPase activity decreased by 20% after incubation with GCA and by 30% with GCA–HRP. Changes of conformation of $Na^+,K^+$-ATPase were detected both after the incubation with GCA and GCA–HRP. These results demonstrate that electronic-excited species inhibit the activity of membrane-bound $Na^+,K^+$-ATPase both by an increase of membrane lateral pressure of the lipid microenvironment of the ATPase molecules and by a direct effect of oxidation of protein residues, i.e. tryptophan, tyrosine.
**Key words:** Electronic-excited species; $Na^+,K^+$-ATPase; Fluorescence.

Generation of electronically excited species of biologically important substances without application of the excitation radiation is extensively studied. It is known that oxidation of 2-methylpropanal (MPAL)* by oxygen present in aqueous solution and with the catalysis by horse-radish peroxidase (HRP) produces the excited acetone in its triplet

* Abbreviations: GCA, glyceraldehyde; TBA, 2-thiobarbituric acid; TCA, trichloroacetic acid; MDA, malondialdehyde; TMADPH, 1-(4-(trimethylamino)phenyl)-6-phenyl-1,2,3-hexatriene; MPAL, 2-methylpropanal; HRP, horse-radish peroxidase (EC 1.11.1.7); $Na^+,K^+$-ATPase, ATP phosphohydrolase (EC 3.6.1.3).

state. Horse-radish peroxidase catalyzes also the aerobic oxidation of Schiff-type adducts of aldehydes and sugars with proteins. These oxidations can generate excited triplet species. The transfer of triplet energy to a suitable acceptor can be utilized for inducing a photochemical reaction, often called "photochemistry without light", which could take place both in plants and animals[1].

Peroxidation is a reaction which can be initiated by excited triplet species. It proceeds through a complex process involving rearrangement and destruction of double bonds of highly unsaturated fatty acids of membrane lipids and oxidation of proteins in biological membrane systems. It is well known that peroxidation of plasma membranes from mammalian cells inhibits the activity of membrane-bound enzymes[2]. Production of malondialdehyde, the dominant secondary product of lipid peroxidation, is a consequence of degradation of polyunsatured fatty acids[3]. Thus, the concentration of MDA can be used as a measure of lipid peroxidation[4].

$Na^+,K^+$-ATPase is a membrane-bound enzyme responsible for the active transport of $Na^+$ and $K^+$ across the plasma membrane of mammalian cells. The enzyme consists of α- and β-subunits, both of which are essential for its function. In the present paper, an inhibition of $Na^+,K^+$-ATPase by enzymically generated excited species is studied. These studies are important, because the inactivation of $Na^+,K^+$-ATPase can lead to various pathological effects in animal organism.

## EXPERIMENTAL

### Chemicals

All the reagents and chemicals used in these experiments were of analytical grade. Tris, ouabain, thiobarbituric acid, glyceraldehyde and trichloroacetic acid were purchased from Sigma Chemical Co. Horse-radish peroxidase were obtained from Serva. TMADPH was purchased from Molecular Probes. 2-Methylpropanal was prepared by oxidation of 2-methyl-1-propanol with a dichromate–sulfuric acid mixture and was purified by destillation.

### Membrane Protein Preparation

$Na^+,K^+$-ATPase (EC 3.6.1.3) from pig kidney outer medulla was prepared by Jørgensen's procedure[5].

### Generation of Triplet Acetone by System MPAL–HRP

Triplet acetone[1] was generated by oxidation of different concentration of 2-methylpropanal (MPAL) in the presence of 0.1 μM horse-radish peroxidase (HRP) in 30 mM Tris-HCl buffer (pH 7.4) for 60 min at 25 °C. The total volume was 1 ml, concentration of membrane protein was 100 μg $ml^{-1}$.

### Incubation of Membrane Protein with GCA and GCA–HRP

Membrane protein samples (100 μg of protein) were incubated in 30 mM Tris-HCl buffer (pH 7.4) for a different time intervals with 10 μM GCA or with 10 μM GCA and 0.1 μM HRP at 25 °C. The total volume was 1 ml, concentration of membrane protein was 100 μg $ml^{-1}$.

Determination of $Na^+,K^+$-ATPase Activity

The rate of ATP hydrolysis was carried out in 1 ml reaction mixture containing 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 3 mM ATP, 30 mM Tris-HCl buffer (pH 7.4) and 20 μg membrane protein. The reaction was carried out at 37 °C for 15 min in the presence and in absence of 2 mM ouabain. The reaction was stopped by the addition of 0.5 ml 12.5% trichloroacetic acid. The samples were kept on ice, centrifuged at 2 000 g for 15 min and the supernatant was analyzed for the inorganic phosphate liberated. The ouabain-sensitive activity of $Na^+,K^+$-ATPase was expressed in % of activity of the control sample[6].

Detection of Malondialdehyde MDA

The solution containing 100 μg of membrane protein, 25 mM TBA, 0.9 M TCA and 50 mM HCl (total volume 2 ml) in a test-tube were heated to 95 °C for 60 min and then vortexed for 15 min with 1-butanol (2 ml). The concentration of MDA was calculated from the absorption ($\varepsilon = 1.56 \cdot 10^5$ l mol$^{-1}$ cm$^{-1}$) of the organic layer at 532 nm with 1-butanol as absorbance blank[4].

Absorption and Steady-State Fluorescence Measurements

Absorption measurements were carried out on a Uvikon 810 spectrophotometer. Steady-state fluorescence data were taken on a Perkin–Elmer LS-5 fluorometer in quartz cuvettes with reflecting walls[7]. All measurements were done at 25 °C.

*Measurements of membrane lateral pressure*. Fluorescence measurement was performed in a cuvette containing 2 μM TMADPH and membrane protein (200 μg). Excitation and emission wavelengths were 365 and 430 nm, respectively. Two Glan–Thompson polarizers were used for detection of steady-state anisotropy values $r_s$. Steady-state fluorescence anisotropy data $r_s$ were recalculated to limiting fluorescence anisotropy values[8] $r_\infty$ (Eq. (*1*))

$$r_\infty = r_0 r_s / (r_0 r_s + (r_0 - r_s)^2 / m) \quad , \tag{1}$$

where $r_s$ is steady-state fluorescence anisotropy, $r_0$ is maximal anisotropy ($r_0 = 0.38$) and $m$ is parameter which expresses the difference between the rotational diffusion of the probe in the membrane and that in the isotropic reference oil[8] ($m = 0.91$). The limiting fluorescence anisotropy $r_\infty$ reflects the range or amplitude of random librational motions of the probe in the membrane. Second rank orientational order parameter data $S_{\delta\infty}$ were calculated from limiting anisotropy values (Eq. (*2*))

$$S_{\delta\infty} = (r_\infty / r_0)^{1/2} \quad , \tag{2}$$

where $r_\infty$ is limiting fluorescence anisotropy and $r_0$ is maximal anisotropy ($r_0 = 0.38$). Membrane lateral pressure was determined from the second rank orientational order parameter values[9] $S_{\delta\infty}$.

*Measurements of tryptophan and bityrosine fluorescence*. To measure tryptophan loss and bityrosine production, membrane protein (200 μg) was added to 30 mM Tris-HCl buffer pH 7.4 (2 ml). Fluorescence emission at 345 nm (295 nm excitation) was used as a reflection of tryptophan content. Bityrosine content was estimated at 325 nm excitation and 410 nm emission in comparison with authentic bityramine and bityrosine[10].

For the analysis of the tryptophan quenching process in $Na^+,K^+$-ATPase we supposed independent, equally absorbing, fluorophores. We considered all the tryptophan residues buried in the membrane to be totally inaccessible to NaI and the others to be accessible with the same value of the Stern–Volmer constant, $K_q$. At such condition the classical Stern–Volmer equation is expressed[11] by Eq. (*3*):

$$F_0/(F_0 - F) = 1/(f_a K_q [Q]) + 1/f_a , \qquad (3)$$

where $F_0$ and $F$ are fluorescence intensities without and with the quencher (NaI), respectively, [Q] is the concentration of the quencher and $f_a$ is the fractional maximum of accessible protein fluorescence. From Eq. (*3*), a plot of $F_0/(F_0 - F)$ vs 1/[Q] yields a straight line of slope $1/(f_a K_q)$ and intercept $1/f_a$.

## RESULTS

### *System MPAL–HRP*

### Inhibition of Activity of $Na^+,K^+$-ATPase

The $Na^+,K^+$-ATPase was incubated with triplet acetone which was generated by oxidation of different concentration of MPAL in the presence of 0.1 μM HRP for 60 min. The transfer of excitation energy of triplet acetone to dissolved oxygen leads to generation of singlet oxygen which initiate peroxidation of biological membranes[12,13]. Inhibition of the activity of $Na^+,K^+$-ATPase and an increase of the level of malondialdehyde were plotted in Fig. 1 as a function of MPAL concentration. Steady-state fluorescence anisotropy of TMADPH was used to determine the membrane lateral pressure[8]. TMADPH is

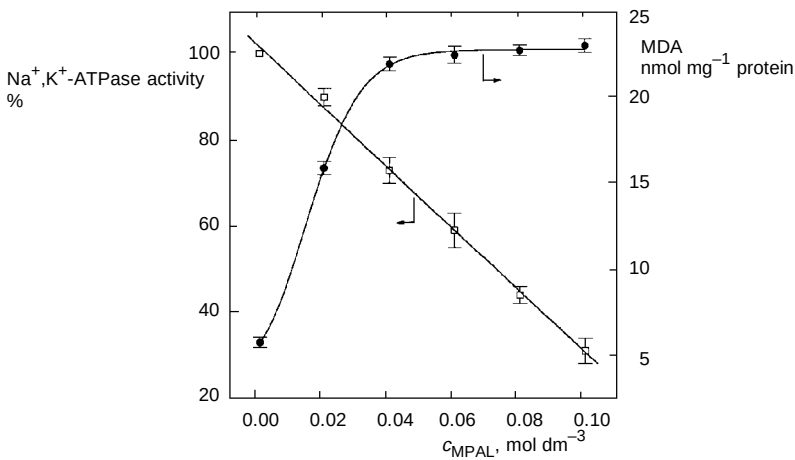


FIG. 1
Inhibition of $Na^+,K^+$-ATPase activity and enhancement of the level of MDA by triplet acetone generated by system MPAL–HRP. The experimental points are average of five independent measurements

a fluorescent probe which is distributed at the membrane–water interface and thus yields information on the effects in that region. Membrane lateral pressure with $Na^+,K^+$-ATPase activity was plotted as a function of MPAL concentration (Fig. 2). The activity of $Na^+,K^+$-ATPase is inhibited by 70% (the concentration of MPAL was 0.1 mol $l^{-1}$).

TABLE I

The loss of native $Na^+,K^+$-ATPase tryptophan fluorescence by triplet acetone. Incubation was done in 30 mM Tris-HCl buffer (pH 7.4), 0.05 M MPAL, 0.1 μM HRP, concentration of protein was 100 μg $ml^{-1}$ at 25 °C. Excitation and emission wavelengths were 295 and 345 nm, respectively

| Incubation time, min | Fluorescence, a.u.[a] |
|---|---|
| 0.0 | 438.5 |
| 0.5 | 298.2 |
| 1.0 | 258.6 |
| 2.5 | 241.1 |
| 5.0 | 212.5 |
| 10.0 | 150.7 |
| 25.0 | 101.6 |

[a] Arbitrary units.

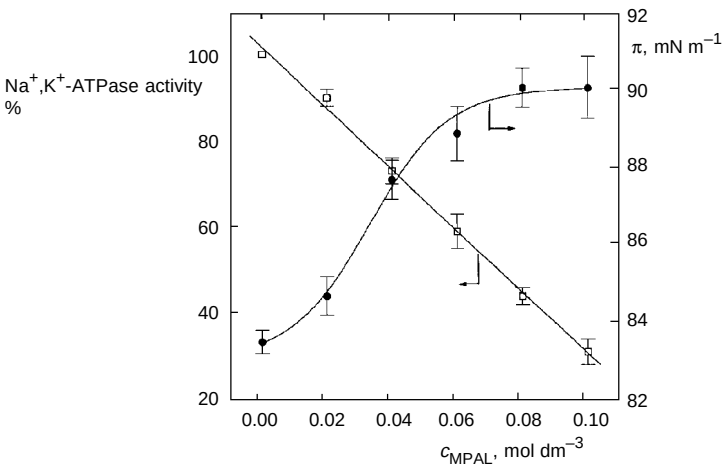


FIG. 2

Inhibition of $Na^+,K^+$-ATPase activity and increasing of the membrane lateral pressure by triplet acetone generated enzymically (HRP) at different concentration of MPAL. Membrane lateral pressure π (in mN $m^{-1}$) was calculated from fluorescence anisotropy of TMADPH (2 μM) in lipid environment of $Na^+,K^+$-ATPase. The experimental points are average of five independent measurements

The concentration of malondialdehyde increased from 5.5 ± 0.5 to 22.6 ± 0.7 nmol $mg^{-1}$ protein (Fig. 2), the membrane lateral pressure increased also from 83.6 ± 0.4 to 90 ± 0.9 mN $m^{-1}$.

## Loss of Tryptophan and Bityrosine Production

$Na^+,K^+$-ATPase contains 16 tryptophans. Exposure of $Na^+,K^+$-ATPase to triplet acetone resulted in the rapid loss of native tryptophan fluorescence (Table I) and elevation of bityrosine production (Table II).

TABLE II

Increase of $Na^+,K^+$-ATPase bityrosine fluorescence during incubation with triplet acetone. The fluorometer cuvette contained $Na^+,K^+$-ATPase 100 μg $ml^{-1}$, 0.05 M MPAL, 0.1 μM HRP. The incubation was done in 30 mM Tris-HCl buffer pH 7.4 at 25 °C. Excitation and emission wavelengths were 325 and 410 nm, respectively

| Incubation time, min | Fluorescence, a.u.[a] |
|---|---|
| 0.0 | 65.4 |
| 15.0 | 88.2 |
| 30.0 | 91.6 |
| 60.0 | 92.8 |
| 90.0 | 95.0 |
| 120.0 | 98.9 |
| 150.0 | 102.6 |
| 180.0 | 108.3 |

[a] Arbitrary units.

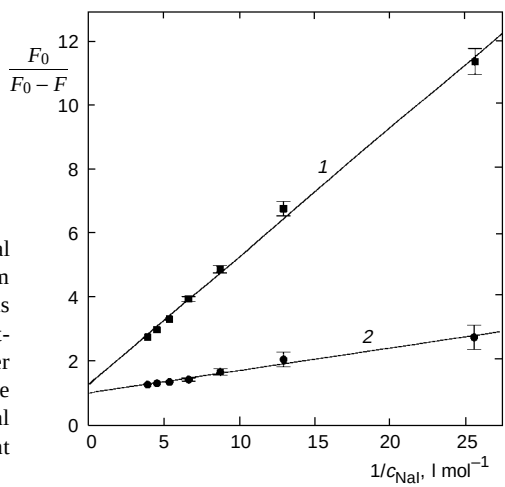


FIG. 3

A modified Stern–Volmer plot of collisional quenching of tryptophan residues from $Na^+,K^+$-ATPase incubated with MPAL plus HRP with NaI as a quencher. *1* Before treatment with the system MPAL–HRP, *2* after 60 min treatment with 0.05 M MPAL in the presence of 0.1 μM HRP. The experimental points are average of four independent measurements

Protein Structural Rearrangement

To obtain information about conformational changes, we followed the collisional quenching of tryptophan residues with NaI. Modified Stern–Volmer plots of quenching before (line *1*) and after treatment of the $Na^+,K^+$-ATPase with triplet acetone (line *2*) are shown in Fig. 3. Table III contains quenching parameters $K_q$ and $f_a$ which were calculated using the modified Stern–Volmer plots.

TABLE III

Accessibility of tryptophan residues of native and treated $Na^+,K^+$-ATPase to iodide quencher. Influence of excited species on the quenching parameters. The quenching parameters $K_q$ and $f_a$ were calculated using the modified Stern–Volmer plots as described in Experimental. Incubation was done in 30 mM Tris-HCl buffer (pH 7.4), concentration of protein was 100 μg $ml^{-1}$ at 25 °C. Total volume was 2 ml. Excitation and emission wavelengths were 295 and 345 nm

| System | $K_q$, $nmol^{-1}$ | $f_a$ |
|---|---|---|
| Native form | 2.08 ± 0.17 | 0.78 ± 0.03 |
| MPAL | 2.12 ± 0.15 | 0.78 ± 0.03 |
| MPAL–HRP | 14.45 ± 0.47 | 1.00 ± 0.03 |
| GCA | 2.83 ± 0.05 | 0.85 ± 0.02 |
| GCA–HRP | 3.09 ± 0.06 | 0.97 ± 0.02 |

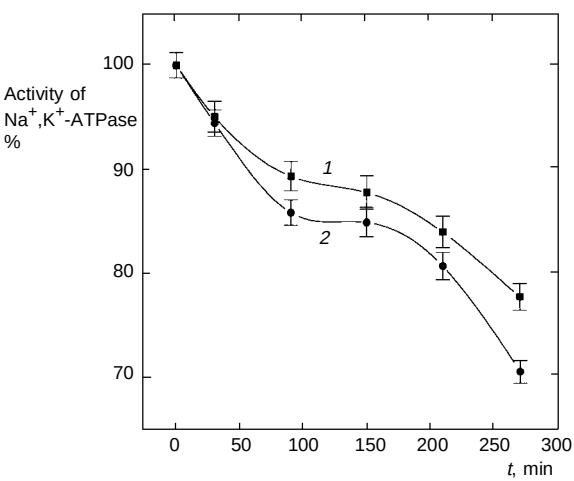


FIG. 4

Inhibition of $Na^+,K^+$-ATPase activity during incubation with GCA (*1*) and GCA–HRP (*2*). The experimental points are average of four independent measurements

*System GCA–HRP*

Inhibition of Activity of $Na^+,K^+$-ATPase

The $Na^+,K^+$-ATPase was incubated with 10 μM glyceraldehyde (GCA) and with system 10 μM GCA–0.1 μM HRP for a different time intervals. Inhibition of activity of $Na^+,K^+$-ATPase by nonenzymatic glycosylation is plotted in Fig. 4 (curve *1*). The activity of $Na^+,K^+$-ATPase is inhibited by 20%. When HRP was present, the activity of $Na^+,K^+$-ATPase decreased by 30% (Fig. 4, curve *2*).

TABLE IV
The loss of native $Na^+,K^+$-ATPase tryptophan fluorescence during the incubation with GCA–HRP. Incubation was done in 30 mM Tris-HCl buffer (pH 7.4), 10 μM GCA, 0.1 μM HRP, concentration of protein was 100 μg $ml^{-1}$ at 25 °C. Excitation and emission wavelengths were 295 and 345 nm, respectively

| Incubation time, min | Fluorescence, a.u.[a] |
|---|---|
| 0.0 | 228.5 |
| 20.0 | 186.3 |
| 40.0 | 178.5 |
| 80.0 | 172.1 |
| 160.0 | 155.7 |
| 240.0 | 147.6 |

[a] Arbitrary units.

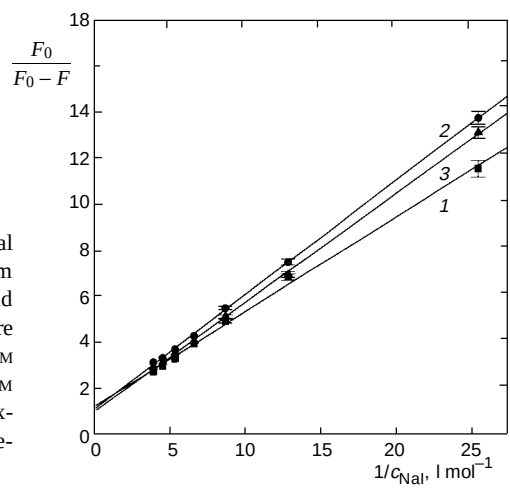


FIG. 5
A modified Stern–Volmer plot of collisional quenching of tryptophan residues from $Na^+,K^+$-ATPase incubated with GCA and GCA–HRP with NaI as a quencher. *1* Before treatment, *2* after 4 h treatment with 10 μM GCA, *3* after 4 h treatment with 10 μM GCA in the presence 0.1 μM HRP. The experimental points are average of four independent measurements

Loss of Tryptophan and Bityrosine Production

No amino acids loss or bityrosine production occurred with exposure to GCA alone, but incubation with system GCA–HRP leads to tryptophan loss (Table IV).

Protein Structural Rearrangement

Modified Stern–Volmer plots of quenching after treatment of the $Na^+,K^+$-ATPase with GCA and with GCA–HRP are shown in Fig. 5. Quenching parameters $K_q$ and $f_a$ are given in Table III.

## DISCUSSION

*System MPAL–HRP*

The results of this study suggest that the inhibition of the membrane-bound $Na^+,K^+$-ATPase activity by the treatment with enzymically generated electronic-excited species, i.e. triplet acetone, may be due to lipid peroxidation of the lipid microenvironment and oxidative modification of protein. Lipid peroxidation is a very complex process. It is likely that the inhibition of the $Na^+,K^+$-ATPase activity is the result of a combination of direct and indirect effects on the lipid microenvironment as well as on the protein molecule itself.

As shown in Fig. 1, incubation of membranes with MPAL in the presence of HRP resulted in a marked inhibition of $Na^+,K^+$-ATPase activity and enhancement of the concentration of MDA. Incubation with MPAL in the absence of HRP resulted in no changes.

The lipid microenvironment where the $Na^+,K^+$-ATPase protomers are inserted is widely known to participate in the modulation of the activity of this enzyme[14,15]. The peroxidation of the fatty acid residues of the lipid microenvironment might participate in the inhibition of this enzyme. Some of the products of lipid peroxidation, i.e. malondialdehyde (MDA), are cross-linking agents of membrane components[16]. The lipid peroxidation, through the formation of Schiff bases, could induce intermolecular cross-links, with the formation of oligomer forms of proteins.

It is well known that lipid peroxidation of plasma membranes produces a decrease of their fluidity[17]. Decreasing of the membrane fluidity can subsequently influence membrane-bound proteins, resulting in their changed structural and functional properties. We used steady-state fluorescence anisotropy of TMADPH to determine the membrane lateral pressure. Increase of the membrane lateral pressure (decrease of membrane fluidity) is shown in Fig. 2. It may be supposed that the increase of membrane lateral pressure can influence the protein conformation[18]. Therefore it seems that alteration in $Na^+,K^+$-ATPase activity induced by lipid peroxidation is partly related to modification

of the ATPase molecules through changes in lipid–protein interactions due to alterations of the lipid environment around the enzyme.

As shown in Tables I and II electronic-excited species can modify protein primary structure. Modification of membranes with triplet acetone leads to tryptophan loss and bityrosine production. Tyrosyl (phenoxyl) radicals are produced as a result of interactions of tyrosyl residues with singlet oxygen. Tyrosyl radicals may then react with other tyrosyl radicals or with tyrosine molecules to form several stable biphenolic compounds. Bityrosine, a 2,2′-biphenol, appears to be the major product[10]. Such oxidative modifications of primary structure underlie the alteration of secondary and tertiary structure[19].

The collisional quenching of protein tryptophan residues by NaI showed that after treatment with triplet acetone 100% of tryptophan residues are fully accessible to NaI, compared with 78% of residues before treatment. Triplet acetone may induce a conformational change of enzyme which alters the accessibility of tryptophan residues.

*System GCA–HRP*

Incubation of membranes with GCA in the presence and in the absence of HRP resulted in a inhibition of $Na^+,K^+$-ATPase activity (Fig. 4). No increasing of concentration of malondialdehyde was observed. Schiff-type $Na^+,K^+$-ATPase adducts with GCA may be responsible for inhibitional effects[20] of GCA. The presence of HRP in reaction mixture leads to higher inhibition of $Na^+,K^+$-ATPase activity and destruction of tryptophan residues. Medeiros and Bechara[21] reported that protein Schiff-type adducts generate electronic-excited species by an aerobic oxidation. Therefore it seems that HRP catalyzes the aerobic oxidation of $Na^+,K^+$-ATPase Schiff-type adducts with GCA and generates electronic-excited species which may be responsible for tryptophan loss (Table IV). No bityrosine production occurred during incubation with GCA or GCA–HRP.

The collisional quenching of protein tryptophan residues by NaI showed that after treatment with GCA 85% of tryptophan residues are fully accessible to NaI, compared with 78% of residues before treatment. After incubation with GCA–HRP 97% of tryptophan residues are accessible to NaI (Table III). Therefore it seems that GCA and system GCA–HRP may induce a conformational change of enzyme which alters the accessibility of tryptophan residues.

In conclusion, this study shows that enzymically generated electronic-excited species (triplet acetone) can induce lipid peroxidation of the lipid microenvironment of $Na^+,K^+$-ATPase and oxidatively modify primary structure of $Na^+,K^+$-ATPase. Glyceraldehyde can form Schiff-type adducts with $Na^+,K^+$-ATPase which influence the ATPase activity. Protein Schiff-type adducts generate electronic-excited triplet species which undergo aerobic oxidation. These processes could explain deleterious effects of aldehydes and sugars by the excited species being the actual toxic agent. Highly reactive and long-lived enzyme generated triplet species can interact with a large number of biomolecules[1].

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