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Effect of the laser irradiation on the functional activity of enzymes with different structural complexity

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ABSTRACT

Three enzymes differing in their structural composition were irradiated by UV lasers to study the effect of temperature, protein concentration and addition of small molecules on their sensitivity to radiation exposure. The laser - induced effects were due to the structural complexity of the protein molecules and depended on the dose applied, the wavelength and the density of irradiation. The multi-enzyme 2-oxoglutarate dehydrogenase complex (2OGDC) was subjected to pronounced irradiation - induced changes whereas the response of the two other enzymes was less significant. Reduction of the protein levels in irradiated samples was important under the XeCl laser coercion and the effects depended on the doses applied. The laser irradiation effects are suggested to be realized by means of conformational changes in the protein molecules and intermolecular association - dissociation processes.

Keywords: UV laser irradiation, 2-oxoglutarate dehydrogenase complex, fluorescence.

1. INTRODUCTION

Laser irradiation of enzymes causes significant changes in their activity. It has been shown that a chromophore - assisted laser inactivates 93% of β-galactosidase activity and 80% of alkaline phosphatase as well as 87% of acetylcholinesterase activity. Although thermal denaturation and photochemical mechanisms for proteins inactivation were postulated[1], the precise nature of the laser - mediated damage to the protein function has not been established.

Three enzymes differing in the complexity of their structural compositions were studied in connection with laser- induced changes in their functional activities. Thiamine triphosphatase (ThTPase: EC 3.6.1.28), characterized by the simplest structure presented by one polypeptide chain (Mr 28 000), alcohol oxidase (AO: EC 1.1.3.13), built of four identical subunits (Mr 300 000) having coenzyme FAD (flavine adenine dinucleotide) in its composition, and 2 -oxoglutarate dehydrogenase complex as an ensemble of three separate enzymes: 2 - oxoglutarate dehydrogenase (E1o: EC 1.2.4.2) , dihydrolipoamide succinyltransferase (E2o:EC 2.3.1.61) and dihydrolipoamide dehydrogenase (E3 : EC 1.8.1.4) , possessing TPP, lipoic acid residues and FAD incorporated as cofactors into E1o, E2o and E3, accordingly, have been examined for their activity under the effect of the UV laser irradiation.

The fluorescence of 2-oxoglutarate dehydrogenase complex from bovine heart after the irradiation by the XeCl and the nitrogen lasers was studied. The fluorescence intensity of bound flavine adenine dinucleotide was increased after high doses of the excimer laser treatment.

2. MATERIALS AND METHODS

The sources of coherent UV irradiation were a pulse nitrogen laser and an excimer XeCl laser. The electron discharging XeCl laser designed at the Grodno State University served as a source of UV irradiation (λ =308 nm). The laser performs

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the following parameters: the generating energy of the pulse was around 2J with a pulse frequency of 1 Hz and halfwidth equal to 50 ns. The specially designed XeCl laser enables us to vary the pulse duration from 25 to 100 ns with generating energy of 0.6 mJ. The protein samples were subjected to laser pulses with energy of 25, 50 and 150 mJ and 60 ns halfwidth. The pulse was generated by separation of the laser beam producing the energy required in the experiment. The beam was focused with a long - focal quartz lens (F=1m) and directed vertically on the tubes containing protein solutions to obtain a spot on the sample with diameter of 5 mm.

Standard nitrogen laser (LGP - 505) was used as source of the UV irradiation (λ=337 nm) to generate a pulsed laser beam with a pulse width of 10ns at a frequency ranging from 12 to 1000 Hz. The protein samples were subjected to 1 min, 3 min and 5 min of laser pulses of 0.25 mJ per pulse at a frequency of 16 Hz and a power 4 MW that corresponded to the doses of irradiation equal to 240 mJ, 720 mJ and 1200 mJ. Use of different doses of irradiation by the two lasers was caused by the facts that eximeric laser generates twofold higher pulse power then nitrogen one and the activity of the XeCl laser emanation was higher. The experiments done have shown that the lowest dose of nitrogen laser irradiation when some changes of the functional activity of 2OGDC become noticeable corresponded to the 1 min of the protein complex treatment.

2OGDC was extracted from bovine heart and purified by the method of Stanley[2] with our modifications[3]. Enzyme activity was assayed by monitoring NADN formation at 340nm and 30°C, alcohol oxidase was isolated from Candida boidinii cells by a specially developed method[4] and thiamine triphosphatase was purified by a method developed for the enzyme from bovine brain[5]. The computations were performed by using linear least square regression[6]. The kinetic parameters were calculated using Lineweaver- Burk plots[7].

Steady-state fluorescence spectra of the protein solutions were recorded by the SDL-2 and DFS-52 spectrometers (LOMO, Russia). Fluorescence decay measurements were performed with a nanosecond pulse fluorimeter [8]. The samples were excited using a nitrogen flash lamp generating exciting pulse with a half width of 1 ns. A PC 486DX was used for the operation and data processing. The decay data analysis (deconvolution) was performed according to the method taking into account the finite width of the profiles of the excitation pulse and the response function of the apparatus.[9,10]

Experimentally obtained fluorescence decay curve I(t) is considered as a convolution of the decay law of the sample F(t) with the instrument response function L(t) [11,12]

$$I(t) = \int_0^t L(t')F(t-t')dt' \qquad (1)$$

The fluorescence decay law is usually simulated as a sum of exponentials

$$F(t) = \sum_j \alpha_j \exp\left(-\frac{t}{\tau_j}\right) \qquad (2)$$

where α_j, and τ_j are the decay amplitude and lifetime respectively.

Using non-linear least squares method the fitting parameters α_j, τ_j were determined by minimizing χ^2_ν [13],

$$\chi^2_\nu = \frac{1}{\nu}\sum_i W(t_i)\cdot\left[I(t_i) - Y(t_i)\right]^2, \qquad (3)$$

where the index *i* sums over the appropriate channel region. $I(t_i)$ and $Y(t_i)$ denote, respectively, the observed and calculated values corresponding to the *i* channel, and $W(t_i)$ is the corresponding statistical weight. ν represents the number of degrees of freedom for the fluorescence decay surface.
$W(t_i)$ is defined as a magnitude, reciprocal to the variance of the observed fluorescence intensity in *i* channel.

$$\frac{1}{W(t_i)} = var\, I(t_i). \qquad (4)$$

We used a modified expression for statistical weights to take into account the influence of noise on the instrument response function L(t).

$$\frac{1}{W(t_i)} = var\, I(t_i) + \int_0^{t_i} var\, L(t')\cdot F^2(t_i - t')dt'. \qquad (5)$$

This modification is useful when the average lifetime is less then or equal to the excitation pulse duration.

To monitor the dependence of the apparatus response function on the wavelength a "shift method"[11] was used.

The protein samples of OGDC, AO and ThTPase were prepared by dissolving of the protein either in 0.02 M phosphate or Tris - buffer (pH 7.4).

3. RESULTS AND DISCUSSION

The radiation studies carried on purified enzymes were performed using an UV laser equipment generating 308 and 337 nm laser light. All the irradiation procedures were performed at 4° C (excluding the experiments on increasing temperature effects). The possible effects of the protein concentration in irradiated sample, temperature and the addition of small molecules- dithiothreitol and sodium azide (NaN_3) on the functional activity of three enzymes of interest-were examined.

Thiamine triphosphatase possessing the simplest composition was unaffected by any of the lasers used. The response of alcohol oxidase, a tetrameric flavoprotein, to excimer laser light exposure was noticeable. It was established that the effects obtained depended on the protein concentration It was shown that when the protein level in the sample was equal to 0.25 mg/ml the laser irradiation at a dose of 50 and 200 mJ diminished the AO activity in both the cases. The kinetic investigations provide evidence for the V_{max} being 80% of the initial value and the Km did not change. In increasing AO concentration to 0.5 mg/ml the effect of these dose irradiation was not detected. However, soon after the treatment of more concentrated preparations (after storing of the enzyme during 24 hours) the AO activity decreased quickly. The preliminary introduction of the alcohol oxidase stabiliser - NaN_3 - to the alcohol oxidase solution did not only protect AO from inactivation but prolonged the enzyme lifetime. The further increasing of the AO concentration to 1 mg/ml led to some activation of the enzyme (up to 130%).

Being the ensemble of tree constituent enzymes, the multi - enzyme 2-oxoglutarate dehydrogenase complex was mostly affected by UV laser irradiation. It has been shown that nitrogen laser caused a significant decrease of the OGDC activity (Table 1). The effect did not depend on the protein concentration in the preparations of the complex. The irradiation of the enzyme solution at 24°C resulted in a loss of 12% of its activity even in the case of short (1min) treatment by the beam possessing medium power of the pulse (720 mJ).

The effect of the temperature increase was unnoticed with using of XeCl laser, whereas the protein level was an important factor. The high dose of the laser irradiation (150 mJ) and reduced protein concentration (up to 0.12 mg/ml) caused a pronounced loss of the OGDC activity (see Table 1). Even lower doses of the laser irradiation resulted in marked inhibition of the diluted preparation of the complex.

Table 1. Activity of the 2-oxoglutarate dehydrogenase complex affected by different doses of UV laser irradiation (t=4°C)

Enzyme concentrations (mg/ml)	ACTIVITY, %					
	XeCl laser (mJ)			Nitrogen laser (mJ)		
	25	50	150	240	720	1200
2OGDC, 0.7	114	108	104	100	108	60
2OGDC, 0.7 + 0.5 mM DTT	-	128	118	108	112	100
2OGDC, 0.25	156	100	-	100	100	70
2OGDC, 0.12	40	40	20	-	-	-
2OGDC, 0.12 + 0.5 mM DTT	100	128	118	-	-	-

The inhibition of the complex activity after the treatment of the enzyme by high doses of both the lasers used in the experiments was almost abolished when a reducing agent dithiotreitol at a concentration of 0.5 mM was presented in the OGDC solution, and even some activation was noticed after the complex was subjected to excimer and nitrogen lasers irradiation. The effect was characteristic both for more concentrated preparations and diluted ones .Interestingly, the low doses of XeCl laser irradiation (25 mJ) resulted in activation of the OGDC in preparations with lower (0.25 mg/ml) protein concentration.

The 2 -oxoglutarate dehydrogenase complex isolated from bovine heart possesses two types of active sites with different substrate affinity, the property we have reported earlier[14] . We therefore ran an experiment to test the effect of the type of laser irradiation and the dose applied on the kinetics of 2OGDC and possible changes in the $S_{0.5}$ and V_{max} values were determined. The low doses of the XeCl laser (25 mJ) applied for diluted preparations of the complex produced a 3-fold decrease of the $S_{0.5}$ values estimated for the slowly functioning active sites localized on the OGD dimers (from 250 to 33 mM), simultaneously increasing this index calculated for the sites with high affinity to 2-oxoglutarate (from 9 to 14 mM) and producing a 100% increase of the V_{max} of the catalysis by these type of active sites. The effects obtained on the concentrated 2OGDC were realized by the mean of $S_{0.5}$ values decreasing determined for quickly - reacting sites (20%) without noticeable changes in the V_{max}.

The inhibition of OGDC activity by nitrogen laser irradiation was attended by 7.5 - fold increase of the $S_{0.5}$ values determined for the active sites binding 2 - oxoglutarate with high affinity and by 2 - fold decrease of V_{max}. Thus, the laser irradiation affects the affinity of the OGD to the substrate as well as the velocity of the reaction of 2 - oxoglutarate decarboxylation.

The third component of 2OGDC, dihydrolipoamide dehydrogenase, catalysing the terminating reaction of the complex, was more sensitive to the effects of irradiation. The treatment of E 3 by high doses of the nitrogen laser (1200mJ) inhibited 50% of the enzyme activity. The excimer laser effects on the activity of the third component of OGDC depended on the dose of irradiation applied, and the most significant inhibition (45%) was registered in response to the protein irradiation by 308 nm laser light with a dose of 150 mJ.

It is suggested that the radiation - caused changes of multi - enzyme complex functional activity can occur at least partially as a result of changing of conformational states of OGDC protein molecules . Intermolecular association - dissociation processes may also take place. The mechanisms can be realized via breaking of the intersubunit interactions after a high dose irradiation. Radiation damage to protein can also occur by free radical reactions as a result of free radical attacks destroying subunits of the enzymes and producing unexpected target sizes of some protein[15]. So DTT protecting OGDC from a loss of activity can act as a free radical scavenger.

The next step of the work was focused on the study of the laser irradiation influence on the fluorescence of tryptophane residues and bound flavine dinucleotide in order to check whether 2OGDC protein molecules underwent some conformational rearrangements induced by the irradiation.

2OGDC was shown to possess significant intrinsic fluorescence (the main parameters of it are listed in the Table 2). Excited at 295 nm, in the region of tryptophane residues absorption, the protein complex exhibits fluorescence with a maximum at 330 nm. This short-wave position of the spectrum may be a result of tryptophane residues localization in low- polar regions of the protein globule, which does not enable water to penetrate. The other possible reason is a significant slowing of relaxation processes in chromophores after excitation. The polarization degree P, determined by segmental mobility of amino acid residues, is characterized by low values (around 0.12), confirming our first suggestion. Obtained small long-wave shift of spectrum (~2 nm, see Table 2) when using "red edge excitation" indicates microenvironment heterogeneity of the excited tryptophanyls.

Table 2. Parameters of 2-OGDC intrinsic fluorescence. λ_{ex}=295 nm. Protein concentration was 0.3 mg/ml.

Sample	Intensity	λ_{max}, nm	P	$\varphi^{3)}$
2-OGDC	1.00	330	0.12	0.050
2- OGDC [1)]	-	332	0.13	-
2- OGDC [2)]	0.90	330	0.12	0.045

[1)] λ_{ex} = 305 nm
[2)] after the XeCl laser irradiation (150 mJ)
[3)] quantum yield was determined relatively to tryptophane with φ=0.20.

The fluorescence decay was nonexponential (Table 3, Fig.1) and might be described by three exponential terms with fluorescence lifetime τ_1= 0.89 ns, τ_2= 2.6 ns, τ_3= 5.4 ns under conditions of satisfactory values for statistical criteria.

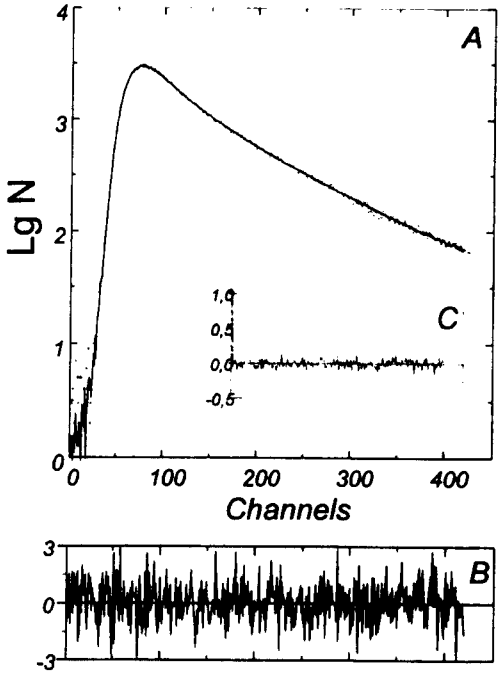

Figure 1. Fluorescence decay curve of 2OGDC. A- experimental and calculated decay curve: dots correspond to experimental data, solid curve is the result of approximation with three-exponential function; B- distribution of weighted residuals; C- autocorrelation function of weighted residuals.

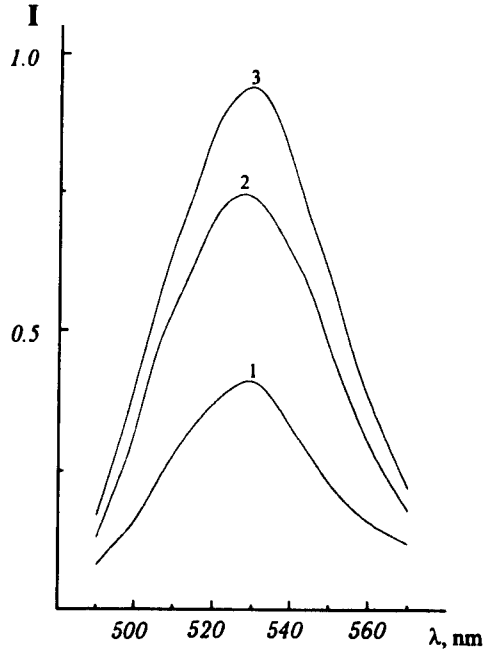

Figure 2. Fluorescence spectra of native 2OGDC (1) and after the XeCl laser irradiation using the doses of 25 (2) and 150 mJ (3).

Table 3. Fluorescence decay lifetimes of OGDC approximated by single, bi-, and three-exponential functions. λ_{ex}= 297 nm, λ_{em}= 340 nm. Standard deviation of τ determination did not exceed 5%. $\langle\tau\rangle = \alpha_1\tau_1+\alpha_2\tau_2+\alpha_3\tau_3$, $(\alpha_1+\alpha_2+\alpha_3=1)$

α_1	τ_1, ns	α_2	τ_2, ns	α_3	τ_3, ns	$\langle\tau\rangle$, ns	χ^2
1.00	3.30	-	-	-	-	-	13.8
0.37	1.20	0.63	4.60	-	-	-	1.04
0.48	0.88	0.31	2.64	0.21	5.38	2.40	0.97

Nonexponential character of tryptophane fluorescence decay is a typical property of proteins[12]. Most of the studied proteins demonstrate the same character of the intrinsic fluorescence decay presented as a sum of three exponentials. There may be three equilibrated conformations of tryptophanyls in respect to a peptide bond where they possess different values for the fluorescence decay constants. The protein structure is not static and undergoes significant fluctuations[16]. As a result, the function of decay is occasionally presented by three continuous chromophore distribution according to the constants of emissive transition[17]. Such distributions are frequently wide enough and are overlapped, and only one or two chromophore distributions can be determined using mathematical calculations[18]. Solving the problem of the distribution type is a

complicated task and need further experiments. The data obtained from the fluorescence decay curves confirm the assumption about high heterogeneity of microenvironment of emitting tryptophanyls.

2OGDC comprises the incorporated FAD as a coenzyme which possesses the property of intrinsic fluorescence with a maximum of the spectrum at 530 nm (Fig.2). The fluorescence intensity of the native protein is low and is equal only to 1% of the free coenzyme fluorescence recorded under the same conditions.

The XeCl laser irradiation of the protein resulted in some decrease of tryptophane fluorescence of the 2-oxoglutarate dehydrogenase complex (Table 2) producing no changes in the emission spectrum, its localization and polarization degree. The FAD fluorescence was more sensitive to the irradiation. The FAD emission intensity increased twofold when treated by a high dose (150 mJ) of the excimer laser (Fig.2).

The data obtained indicate that the active site localized on the E3 component of 2OGDC had a pronounced sensitivity to the XeCl laser irradiation. These results are in agreement with the kinetic studies which showed a loss of this constituent enzyme activity depending on the irradiation dose applied. It is known that the fluorescence of FAD incorporated into the protein is effectively quenched by the neighboring amino acid residues (tyrosine, tryptophane or cysteine) possessing groups which can be electron donors[16]. The laser irradiation causes conformational changes in the enzyme structure, thus affecting the structure of the active site. These processes resulted in decreased quenching and enhanced fluorescence of the protein molecule. Moreover, one or several tryptophane residues possibly localized in the vicinity of the active site contribute to the changes in the protein intrinsic fluorescence. Since 2OGDC is a polytryptophane protein, we have not found noticeable changes in a number of fluorescence parameters, such as spectrum localization, polarization degree and others.

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