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# DEPENDENCE OF PROLONGED AFTERLUMINESCENCE OF PROTEINS ON THE SECONDARY STRUCTURE OF THEIR MOLECULES

[Following is the translation of an article by S. L. Aksentsev, S. D. Nisenbaum, and S. V. Konev, Laboratory of Biophysics and Isotopes, AN BSSR, Minsk, published in the Russian-language periodical Biofizika (Biophysics) 1968, Vol XIII, No 3, pp 428—432. It was submitted on 1 Sep 1967.]

In a previous work [1] we showed that the structural transitions which take place in molecules of pepsin in the acid and alkaline pH range cause a change in the kinetics of attenuation of afterluminescence of solutions of this protein. But it remained unclear, will the parameters of prolonged afterluminescence of other proteins correlate with changes in the structure of their molecules and, what is most important, during reorganization of what or which levels of structural organization does a change take place in the nature of transformation of energy of light quanta, absorbed by the protein molecule, into energy of prolonged afterluminescence.

In connection with this in the present paper investigations are continued on the influence of the structure of the protein macromolecule on the kinetics of attenuation of prolonged afterluminescence of solutions of proteins.

Measurements were made on a unit and by the method described earlier [1]. In the work we used imported commercial preparations of proteins which had not be subjected to additional purification: serum albumin of a bull (SAB), "Koch-Light Laboratories"; human serum albumin (SACh), "Cyclo Chem. Corp."; pepsin 3X cryst., "Lawson"; egg albumin (YaA)* "VDN"; globin and trypsin, "Spofa"; chymotrypsinogen 3X cryst., lysozyme and trypsin inhibitor, "Reanal".

* The preparation was kindly given to us by I. I. Sapezhinskiy, to whom the authors express their deep thanks.

In concentrations of 0.025—0.2% the proteins were diluted in 0.1 n KCl. Change of pH of the protein solutions was achieved by adding 0.2 n HCl and 0.1 n KOH to them. pH values were controlled with an accuracy to 0.05 units. As agents for changing the structure of the protein macromolecule we used urea, guanidine hydrochloride, an anion detergent Na-dodecylsulfate (NDS), ethylene glycol, and methyl and propyl alcohol. For a quantitative appraisal of change in the parameters of prolonged afterluminescence of proteins

in various structural states we measured the constant (K) of rate of attenuation of afterluminescence in coordinates lg of intensity - time. For K we used a value, reverse to the time of life of free radicals $\tau$. In certain cases we determined the intensity of luminescence in 20 seconds after termination of irradiation ($I_{20}$) and the time for a decrease of I by 2 times ($t_{\frac{1}{2}}$). Calculation of curves was performed on the segment from the beginning of attenuation to approximately 3—4 minutes.

As is known, urea and guanidine are effective agents for despiraling of protein molecules. Therefore first of all a study was made of the influence of these substances on the parameters of afterluminescence.

It is necessary to note the difficulty which developed during measurements of the afterluminescence of proteins with various admixtures due to the luminescence of the admixtures themselves. Assuming that in our case the law of additivity was satisfactorily fulfilled, we obtained the true picture of attenuation of luminescence of the changed protein as the difference in the curves of total luminescence and the luminescence of the admixture.

Table 1

Parameters of afterluminescence of proteins in a native state and after 10 seconds of action of guanidine on them (3M guanidine)

| (a) Белок | K сек$^{-1}$ (b) | | $t_{1/2}$ (сек) (c) | | $I_{20}$ нативного белка (f) / $I_{20}$ белка в гуанидине (g) |
|---|---|---|---|---|---|
| | нативного белка (d) | белка в гуанидине (e) | нативного белка (d) | белка в гуанидине (e) | |
| h, САБ | 0,0038 | 0,0185 | 200 | 10 | 11,7 |
| i, САЧ | 0,0073 | 0,0220 | 72 | 20 | 4,7 |
| j, Трипсин ингибитор | 0,0105 | 0,0134 | 37 | 20 | 1,5 |
| k, Лизоцим | 0,0130 | 0,0175 | 34 | 26,5 | 1,2 |
| l, Химотрипсиноген | 0,0125 | 0,0240 | 30 | 18 | 1,6 |
| m, ЯА | 0,0124 | 0,0136 | 30 | 30 | 1,0 |
| n, Трипсин | 0,0190 | 0,0274 | — | — | 2,3 |
| o, Глобин | 0,0169 | 0,0290 | 17,2 | 9,7 | 3,1 |
| p, Пепсин | 0,024 | 0,048 | — | — | 4,0 |

$I_{20}$ - intensity of luminescence in 20 seconds after termination of irradiation; $t_{\frac{1}{2}}$ - time for decrease of I by two times. Concentration of proteins - 0.1%. All measurements at pH 7 (pepsin - pH 5.5) in 0.1 n KCl.

Key: (a) Protein; (b) K sec$^{-1}$; (c) $t_{\frac{1}{2}}$ (sec); (d) native protein; (e) protein in guanidine; (f) $I_{20}$ of native protein/; (g)/$I_{20}$ of native protein in guanidine; (h) SAB; (i) SACh; (j) Trypsin inhibitor; (k) Lysozyme; (l) Chymotrypsinogen; (m) YaA; (n) Trypsin; (o) Globin; (p) Pepsin.

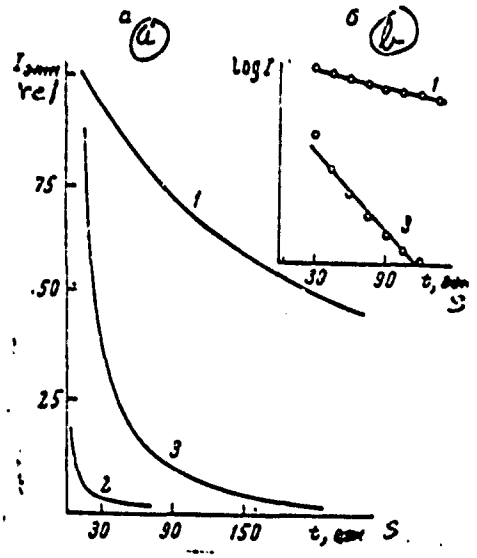


Figure 1. (a) Curves of attenuation of afterluminescence of a 0.025% bull serum albumin in a native state (1) and in a solution of 3M guanidine (2). 3 - attenuation of afterluminescence of 0.1% SAB in 3M guanidine. (b) Anamorphosis of curves of attenuation in coordinates lg I—t.
I - intensity of luminescence in relative units; t - time in seconds. Temperature 20°.

The results of measurements of K, $I_{20}$, and $t_{\frac{1}{2}}$ for solutions of nine proteins in a native state and after a 10-second action of hydrochloride guanidine on them are presented in Table 1. On Fig. 1 is shown the action of guanidine on the parameters of afterluminescence of SAB. All the measurements were made at pH 7 (with the exception of pepsin - pH 5.5). In this series of tests 5 ml of 6M solution of guanidine was mixed with 5 ml of irradiated solution of protein. This eliminated the necessity of taking into consideration of the luminescence of guanidine itself. It can be seen from Table 1 that K of afterluminescence of protein in 3M guanidine in all cases is greater than the corresponding value for native protein, but $I_{20}$ is less. An analogous effect was exerted by solutions of urea. However, it may be thought that the change in the parameters of afterluminescence is caused not by a change in the structure of protein, but by the influence of guanidine and urea on physico-chemical processes, leading to scintillation. In order to check this assumption a study was made of the action of guanidine and urea on glycyl-tryptophan and lactalbumin hydrolyzate (Fig. 2, B). It turned out that neither 8M of urea nor 3M of guanidine changes the K of attenuation of afterluminescence of these substances. A more correct control would be the investigation of the influence of urea or guanidine on a preparation, completely devoid of the characteristic protein

structure, but with the same amino acid composition as in native protein. For obtaining such a preparation the SAB was subjected to proteolysis with pepsin (ratio by weight of pepsin to SAB 1:100). It follows from Fig. 2, A that, first of all, K of the proteolyzate is considerably greater than in native protein, which by itself reflects the influence of structure on the kinetics of attenuation, and , secondly, K of the proteolyzate does not change in a 3M solution of guanidine.

Thus it can be maintained that the change in the parameters of prolongod afterluminescence of proteins following the action of guanidine and urea on them is caused by the disruption of their native structure.

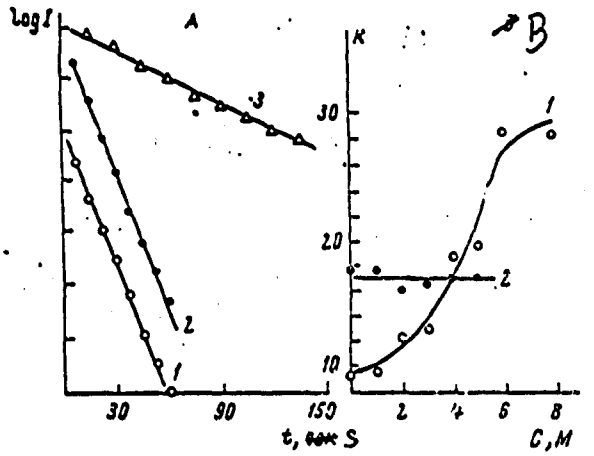


Fig. 2, A. 1 - kinetics of attenuation of afterluminescence of an 0.2% solution of proteolyzate of bull serum albumin (pH 7, 0.1 n KCl); 2 - same in the prosence of 3M solution of guanidine; 3 - attenuation of afterluminescence of native protein; B. 1 - dependence of constant of afterluminescence K (seconds$^{-1}$ · $10^{-3}$) of bull serum albumin on the concentration of urea (pH 7, 0.1 n KCl); 2 - samo for lactalbumin hydrolyzate.

Also in favor of this affirmation is the dependence of attenuation of afterluminescence of SAB on the concentration of urea (Fig. 2, B), which agrees well with the dependence on the concentration of urea, viscosity, and specific optical rotation [2,3]. Just as the changes of viscosity and optical rotation, changes in the parameters of afterluminescence bear a reversible nature. Following a 3-fold dilution of a mixture of proteins--8M urea with 0.1 n solution of KCl, immediately after irradiation K of attenuation turned out to be considerably greater than in native protein. However, the

kinetics of attenuation of the same mixture, recorded 5 minutes after dilution, differed little from the kinetics of attenuation of native protein (Figs. 3, A). In other words, changes of K under the action of urea were reversible as a result of the processes of renaturation of protein, which are completed in several minutes. The time of renaturation was reduced with a lowering of the end concentration of urea in the diluted mixture. Thus, following irradiation of SAB in 6M urea and the subsequent strong dilution of the mixture to a urea concentration of 0.4M, we immediately obtained kinetics of attenuation which are close based on parameters to the afterluminescence of native protein.

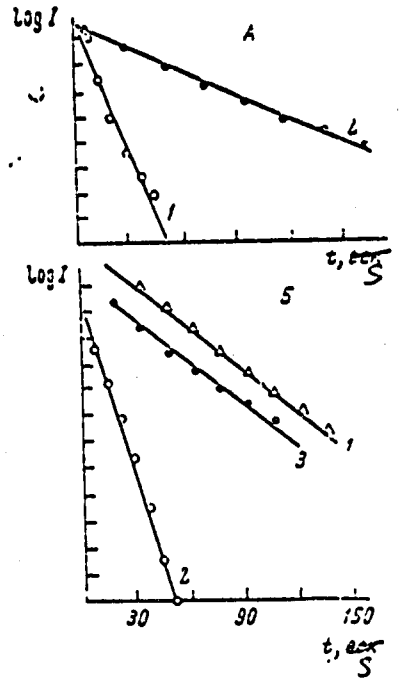


Fig. 3. A - Kinetics of attenuation of afterluminescence of 0.1% solution of bull serum albumin (pH 7, 0.1 n KCl) in urea. The protein - 8M urea mixture was diluted three times after irradiation; 1 - immediately after dilution; 2 - 5 minutes after dilution. B - 1 - Kinetics of attenuation of afterluminescence of 0.05% solution of SAB in water, pH 7 (without addition of KCl); 2 - same in 8M urea; 3 - same after addition of 2 NDS. Ratio of concentrations of detergent to protein 1/10.

The last result and the experiments discussed above (Table 1), in which guanidine was introduced into the solution after irradiation of the protein, indicate that the change in the parameters of afterluminescence is not the result of the possible effect of struc-

ture on the nature of products which are formed in the luminous stage of the photochemical process, but is connected with the influence of structure on events which take place in the course of obscure reactions and are terminated with scintillation

It is interesting to note that various proteins are distinguished not only based on constant of rate of attenuation, but also based on the sensitivity of parameters of afterluminescence to the action of guanidine and urea. At the same time that for SAB the greatest changes of K and $I_{20}$ were observed, the K of YaA under conditions of the experiment was changed insignificantly. If in 4M urea K of afterluminescence of SAB is significantly increased, then for pepsin with the same concentrations of urea K does not change. This agrees with the greater, in comparison with other proteins, stability of the structure of YaA and pepsin to the action of despiralizing agents [3].

Table 2

Influence of spiralizing solutions on parameters of afterluminescence of proteins

| (a) Спирализующий растворитель | (b) Белок | pH | (d) $t_{1/2}$, сек в воде | (e) $t_{1/2}$, сек в спирализующем растворителе | (f) Отношение $I_{20}$ в спирализующем растворителе к $I_{20}$ в воде |
|---|---|---|---|---|---|
| (g) Метиловый спирт 70%-ный+0,01 н. HCl | (c) ЯА | 2,2 | 20 | 60 | 10 |
| | САБ | 2,2 | 3 | 9 | 2,2 |
| (h) Пропиловый спирт 50-ный+0,01 HCl | ЯА | 2,2 | 22 | 69 | 6,7 |
| | САБ | 2,2 | 6 | 21 | 2,6 |
| (i) Этиленгликоль 70%-ный | ЯА | 7,0 | 40 | 60 | 2,2 |
| | САБ | 7,0 | 200 | 70 | 0,43 |

Key: (a) Spiralizing solution; (b) Protein; (c) YaA (on top) and SAB (below); (d) $t_{\frac{1}{2}}$, sec in water; (e) $t_{\frac{1}{2}}$, sec in spiralizing solution; (f) Ratio of $I_{20}$ in spiralizing solution to $I_{20}$ in water; (g) Methyl alcohol 70% + 0.01 n HCl; (h) Propyl alcohol 50% + 0.01 HCl; (i) Ethylene glycol.

It follows from the data cited that breakdown of the secondary and tertiary structure of the protein molecule leads to a drop in the intensity of and an increase in the constant of rate of attenuation of afterluminescence. It could have been expected that agents, exerting an opposite action on secondary structure, i.e., increasing the % of spiralization, cause an opposite effect, decreasing K and increasing the intensity of luminescence. Such an action is possessed, for example, by NDS, which restores the initial values of viscosity and specific optical rotation in proteins which have been denatured with 6M urea [3,4]. We observed an analogous influence of NDS based on parameters of afterluminescence (Fig. 3, B): the addition of 0.5 mg NDS to 5 mg of SAB, denatured with 8M urea, completely restored the value of K, although the intensity of afterluminescence

was not increased to the value which is characteristic for native protein. The effect of the action of NDS did not depend on when it was added to the solution of protein, simultaneous with the urea or after it, since urea already had an effect on the proteins.

From the same point of view there is interest in the action of several low-polarity solutions which disrupt the tertiary structure of a protein and increase the degree of alpha-spiralization of molecules. It is known, for example, that methanol and propanol possess a spiralizing effect. The effect of spiralization is increased in an acid medium [5].

In Table 2 are presented the results of experiments on the measurement of $t_{\frac{1}{2}}$ and $I_{20}$ for SAB and YaA in methyl and propyl alcohols at pH 2.2 and in ethylene glycol at pH 7.0, and on Fig. 4 are depicted the curves of attenuation of YaA in water and in methyl alcohol.

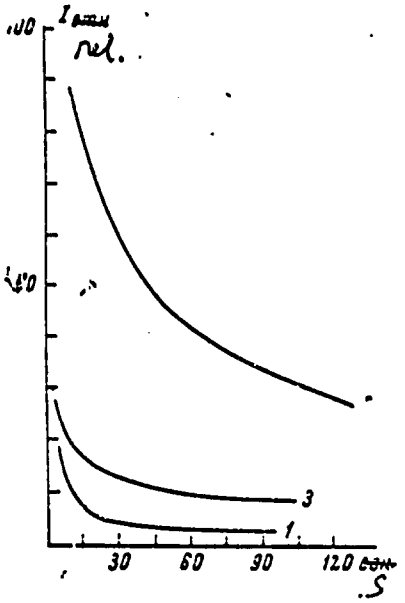


Fig. 4. 1 - curve of attenuation of afterluminescence of 0.1% egg albumin at pH 2.2; 2 - curve of attenuation of a mixture of egg albumin — 70% methyl alcohol (pH 2.2); 3 - afterluminescence of solvent (70% methyl alcohol at pH 2.2).

In acid aqueous solutions of SAB and YaA it was not possible to measure K due to the low intensity of afterluminescence, therefore it was necessary to be limited to the measuring of $t_{\frac{1}{2}}$.

In conformity with data on an increase of spirality of proteins in methanol and propanol at pH 2.2, we observed a considerable increase of $I_{20}$ and $t_{\frac{1}{2}}$. Changes of those parameters in ethylene glycol at pH 2.2 could not be detected. Attention is drawn to the fact that the action of ethylene glycol on SAB and YaA at pH 7 bore an opposite nature. The lessoning of $t_{\frac{1}{2}}$ and $I_{20}$ in SAB under these conditions is apparently connected with the great change in the tertiary structure of the protein molecule (in comparison with secondary): in weakly polar solvents the tertiary structure of these proteins is partially disrupted, and the effect of spiralization in SAB, in contrast to YaA, is insignificant /6/.

However, it seems to us that the secondary structure makes a greater contribution to the values of K, $I_{20}$, and $t_{\frac{1}{2}}$ than tertiary. This is testified to by the fact that in solvents with little polarity in an acid medium, where the tertiary structure is disrupted, an increase of spiralization leads, nevertheless, to a decrease of K and amplification of intensity of afterluminescence. The question concerning the influence of secondary and tertiary structure of a protein on the parameters of afterluminescence requires further investigations.

## Conclusions

1. Investigations were made of the parameters of a number of proteins in a native state, in solutions of urea, guanidine dodecylsulfate, and in weakly polar solvents.

2. Unfolding of the secondary structure of macromolecules of protein is accompanied by an increase in the constant of rate of attenuation and a lessening of intensity of luminescence.

3. An increase, under the influence of dodecylsulfate, of the degree of spiralization of protein which has been denatured by urea leads to restoration of the value of K to values which are characteristic for native protein.

4. An increase of degree of spiralization with a simultaneous disruption of tertiary structure leads to an increase in intensity and decrease of the constant of the rate of attenuation of afterluminescence.

5. The structure of a macromolecule exerts a predominant influence on the parameters of afterluminescence of proteins in the course of obscure processes which begin after cessation of irradiation and lead to scintillation.

6. To a certain degree the parameters of afterluminescence of proteins reflect the degree of alpha-spiralization of their polypeptide chains.

## Literature


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