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**THE RATE CONSTANTS OF THE REACTION OF HYDROXYL
RADICALS ($^{\circ}\text{OH}$) WITH ALCOHOL DEHYDROGENASE AND
GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE**

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Abstract: The rate constants of the reactions of alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase with hydroxyl radicals were determined using the method of steady-state competitive reactions. Ethanol was used as a scavenger of hydroxyl radicals.

The rate constants of the reactions of hydroxyl radicals with alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase were found to be $2.8 \cdot 10^{12} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, and $1.6 \cdot 10^{12} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, respectively.

Key Words: Rate Constant, ADH, GAPDH, Hydroxyl Radical, Ethanol

INTRODUCTION

The rate constants of the reactions of water radicals with proteins are basic parameters in the study of free-radical reactions. The most highly reactive radical, of very high redox potential, is the hydroxyl radical. It demonstrates very strong oxidation characteristics, due to which it reacts very rapidly with most of the compounds occurring in cells and tissues. The main components of cells are water (70%) and proteins ($\approx 20\%$), while DNA only makes up less than 1% of the cell weight. Therefore, most of the radiation-induced reactions in cells are those of primary radicals with proteins. DNA is mainly protected against the indirect effect of radiation by those proteins that scavenge primary radicals. There is also a high probability that protein radicals react further with DNA. The rate constants of the reactions of $^{\circ}\text{OH}$ radicals with proteins have values of the order of 10^9 - $10^{11} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ [1].

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Abbreviations used: ADH – alcohol dehydrogenase; GAPDH – glyceraldehyde-3-phosphate dehydrogenase

The aim of this study was to estimate the rate constant of the reactions of the hydroxyl radical with two enzymes: alcohol dehydrogenase (alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1) and glyceraldehyde-3-phosphate dehydrogenase (D-Glyceraldehyde 3-phosphate:NAD⁺ oxidoreductase, EC 1.2.1.12).

MATERIALS AND METHODS

Materials

We used alcohol dehydrogenase (ADH) with a molecular weight of 147 kDa from baker's yeast, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with a molecular weight of 143 kDa from rabbit muscle; both were of the highest purity grade, and from Sigma (St.Louis, MO, USA). All the other chemicals were of analytical grade and were purchased from POCH (Gliwice, Poland).

Methods and experimental conditions

The rate constants of the reactions of °OH radicals with the enzymes ($k_{(OH + E)}$) and the rate constants for the inactivation of the enzymes (k_i) were determined via the steady-state competitive reaction method [2, 3], measuring the inactivation of enzymes irradiated in the presence and absence of ethanol, which competed with the enzyme for °OH radicals. The concentration of alcohol in the irradiated solutions ranged from 10^{-4} mol·dm⁻³ to 10^{-2} mol·dm⁻³. Protein preparations at a concentration of 0.1 mg·cm⁻³ were irradiated with X rays in phosphate buffer, pH 7.0 in air. The dose rate was 1.5 kGy/h.

ADH activity was measured using the method of Bonnishen and Brink [4] and GAPDH activity was measured using the method of Amelunxen and Carr [5]. The activities of both enzymes were determined from the rate of reduction of NAD⁺ to NADH. The formation of NADH was estimated by measuring the increase in absorbance at $\lambda=340$ nm.

Calculation of the reaction rate constant [2, 3]

The enzyme solutions studied, irradiated under air, are inactivated mainly due to reactions with °OH radicals:

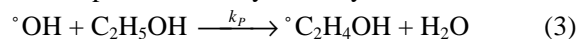


where: k_i denotes the rate constant of the reactions of the °OH radicals with the enzyme leading directly to enzyme inactivation, and [E] is enzyme concentration in mol·dm⁻³.

A fraction of the °OH radicals react with the enzyme without having a significant effect on enzyme inactivation:



If the enzyme solution contains ethanol, which reacts with °OH radicals competitively with respect to the enzyme, enzyme inactivation is attenuated:



Taking into account these reactions of $^{\circ}\text{OH}$, one can assume that the radiation yield of enzyme inactivation equals the fraction of efficiency of $^{\circ}\text{OH}$ radicals causing inactivation [3, 4]:

$$G_{inact.} = \frac{k_i [E]}{k_i [E] + k_E [E] + k_p [P]} G(^{\circ}\text{OH}) \quad (4)$$

where: k_E is the rate constant of the reactions of $^{\circ}\text{OH}$ radicals with the enzyme which do not lead to inactivation, k_p is the rate constant for the reaction of $^{\circ}\text{OH}$ radicals with ethanol ($1.9 \cdot 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$) [1], and $[P]$ is ethanol concentration in $\text{mol} \cdot \text{dm}^{-3}$. In the case of irradiation in the absence of ethanol, the component $k_p [P]$ disappears.

If the enzyme concentration decays exponentially as a function of radiation dose, the radiation yield for inactivation can be determined from the dependence:

$$G_{inact.} = \frac{[E]}{a \cdot D_{37}} \quad (5)$$

where: D_{37} is the radiation dose at which 37% of the enzyme maintains activity, and a is a calculation factor dependent on the units employed ($\alpha = 1.036 \times 10^{-7} [\text{kg dm}^{-3} \text{ mol radical}^{-1} \text{ eV J}^{-1}]$).

Taking into account equations (4) and (5), one can obtain the following dependence:

$$G(^{\circ}\text{OH}) a (D_{37}^P - D_{37}^C) = \frac{k_p}{k_i} [P] \quad (6)$$

in which: D_{37}^P and D_{37}^C denote radiation doses at which 37% of the enzyme maintains activity in the presence and in the absence of the scavenger, respectively, and $G(^{\circ}\text{OH}) = 2.7$.

In the absence of the scavenger:

$$G(^{\circ}\text{OH}) a D_{37} = \left(1 + \frac{k_E}{k_i} \right) \cdot [E] \quad (7)$$

RESULTS

Figs. 1 and 2 show the dependence of the logarithm of relative enzyme activity on the radiation dose for preparations irradiated in the absence and in the presence of ethanol. These dependencies are linear ($r^2 > 0.97$), which indicates that the inactivation reactions of the enzymes studied are of pseudo-first order. Ethanol did not affect the activities of the enzymes studied in the concentration range employed. From the dependencies presented in Figs. 1 and 2, D_{37} values were determined and, on this basis, the dependencies described by Equations 6 and 7 were plotted, and proven to be fairly linear ($r^2 > 0.96$; Figs. 3 and 4).

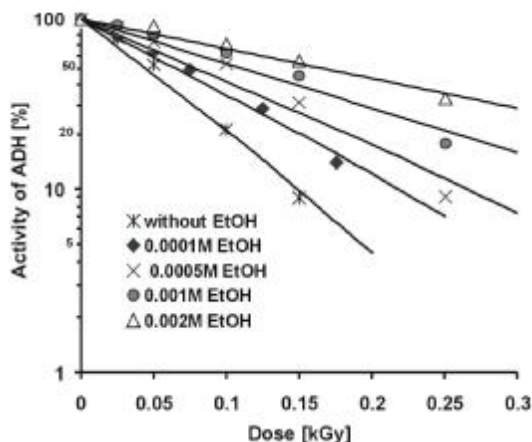


Fig. 1. The dependence of ADH activity on radiation dose ($r^2 > 0.97$).

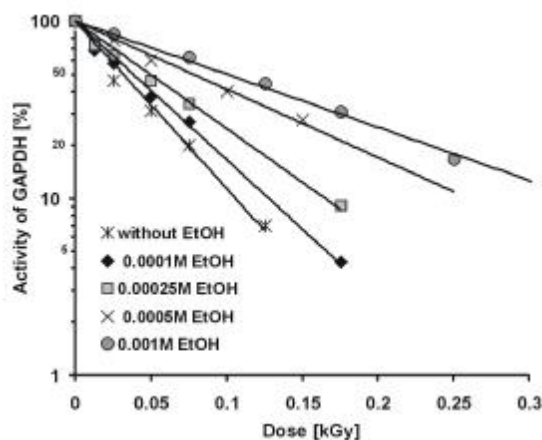


Fig. 2. The dependence of GAPDH activity on radiation dose ($r^2 > 0.97$).

From the dependencies shown in Fig. 3, the rate constants for the reactions of enzyme inactivation by $^{\circ}\text{OH}$ radicals (k_i) were determined. The rate constants for the inactivation of both enzymes are similar and equal to $(7.5 \pm 0.4) \cdot 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ and $(6.3 \pm 0.2) \cdot 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ for ADH and GAPDH, respectively. From the plots presented in Fig. 4, according to dependence (7), the values of the rate constants for reactions not leading to inactivation of the enzymes studied were calculated. They amount to $(2.7 \pm 0.2) \cdot 10^{12} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ and $(1.5 \pm 0.1) \cdot 10^{12} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ for ADH and GAPDH, respectively; i.e. they are respectively 36 and 24 times higher than the rate constants for the reactions of inactivation.

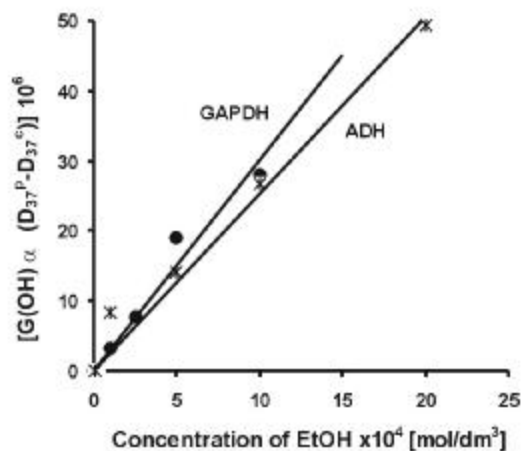


Fig. 3. The effect of ethanol concentration on the inactivation of enzymes ($r^2 > 0.97$).

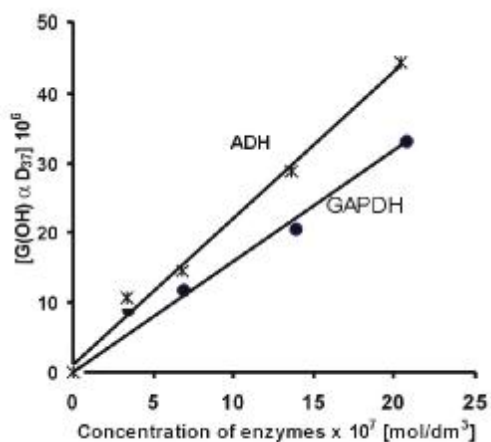


Fig. 4. The dependence (D_{37}) for inactivation as a function of enzyme concentration ($r^2 > 0.98$).

Since the same substrates participate in reactions (1) and (2), the overall rate constant for the reactions of $^{\circ}\text{OH}$ radicals with an enzyme is $k(^{\circ}\text{OH} + \text{E}) = k_i + k_E$. The values of this constant are $2.8 \cdot 10^{12} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ and $1.6 \cdot 10^{12} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ for ADH and GAPDH, respectively.

DISCUSSION

The loss of activity of irradiated enzymes is mainly due to reactions of $^{\circ}\text{OH}$ radicals with amino acid residues located in the active site, i.e. directly engaged in catalysis; e.g. the -SH groups of cysteine and/or some residues of aromatic amino acids (Reaction 1) [6].

Of course, $^{\circ}\text{OH}$ radicals also react with other amino acid residues disposed (located/dispersed) at the surface of an enzyme molecule. These reactions may be followed by the transfer of radicals to other residues, e.g. the more deeply located residues of tyrosine and tryptophan [7]. These reactions can also lead to changes (though apparently to a smaller extent) in protein structure and activity. Therefore, the distinction between the reactions of $^{\circ}\text{OH}$ radicals with an enzyme corresponding to reactions (1) and (2) is to some extent arbitrary; nevertheless, it indicates the fraction of $^{\circ}\text{OH}$ radicals which cause direct inactivation (2.4% and 4% for ADH and GAPDH, respectively). Under aerobic conditions in the absence of ethanol, irradiation of proteins leads also to fragmentation, which contributes to enzyme inactivation [8, 9]. Other products of water radiolysis under aerobic conditions have a negligible contribution to enzyme inactivation. The superoxide radical O_2^- has a 4-5 orders of magnitude lower reactivity with proteins with respect to the hydroxyl radical (e.g. $k(\text{O}_2^- + \text{GAPDH-NADH complex}) = 2 \cdot 10^7 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$; $k(\text{O}_2^- + \text{oxyhemoglobin}) = 4 \cdot 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$; and $k(\text{O}_2^- + \text{ceruloplasmin}) = 5.8 \cdot 10^6 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$) [10]. Moreover, the radiation yield of these radicals is the same in the absence and in the presence of ethanol.

It can also be assumed that the ethanol radicals formed in reaction (3) do not significantly affect enzyme inactivation. The oxygen concentration in the solution ($c(\text{O}_2) \cong 2.8 \cdot 10^{-4} \text{ mol} \cdot \text{dm}^{-3}$) is considerably higher than that of the enzymes studied ($c(\text{E}) \cong 10^{-7} \text{ mol} \cdot \text{dm}^{-3}$), so the majority of the ethanol radicals react with oxygen forming peroxide radicals, which, like superoxide radicals, show a much lower reactivity with proteins [11]. It was assumed that consecutive reactions of $^{\circ}\text{OH}$ radicals with a protein molecule proceed with the same rate constant (i. e. that the previous reactions of $^{\circ}\text{OH}$ radicals do not affect significantly subsequent reactions). Such an assumption could be made, since changes in enzyme activities were determined for relatively low radiation doses, when the logarithms of enzyme activities show a linear dependence on radiation dose ($r^2 > 0.98$); this indicates that the inactivation reaction is of pseudo-first order and the rate of reactions of $^{\circ}\text{OH}$ radicals with enzymes does not change significantly.

The most frequent site of attack of $^{\circ}\text{OH}$ radicals on protein molecules are amino acid residues at the surface of the molecules. The C_α groups of the peptide chain react with $^{\circ}\text{OH}$ radicals with a rate constant of $2 \cdot 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ [12, 13]. The rate constants for the reactions of $^{\circ}\text{OH}$ radicals per one amino acid residue calculated from data obtained in this study were $2 \cdot 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ and $1.2 \cdot 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ for ADH and GAPDH, respectively. Therefore, the size of a protein molecule (surface to volume ratio) is the decisive factor determining the rate constants for the reactions of $^{\circ}\text{OH}$ radicals with proteins. However, the effect of more deeply located residues, especially sulfur and aromatic amino acid, on the rate constant of the protein reaction with $^{\circ}\text{OH}$ cannot be definitely excluded.

These residues may be radical acceptors in the process of electron transfer from the protein surface. First of all, such amino acid residues located in the active site of the enzyme, may significantly affect the reaction rate constant. The active site of ADH contains two Cys residues and one His residue, while that of GAPDH consists of Cys and His. Although the rate constants of the reactions of the enzymes studied with $^{\circ}\text{OH}$ do not differ considerably, the higher content for ADH may be due to the higher content of reactive sulfur and aromatic amino acid residues. The total amount of Cys, Trp and Tyr residues is 27 for ADH and 16 for GAPDH.

The rate constants for enzyme inactivation are usually 1-2 orders of magnitude lower than the overall rate constants for the reactions of $^{\circ}\text{OH}$ radicals with proteins (in this study, 36 times for ADH and 24 times for GAPDH). This is due to the fact that a large fraction of $^{\circ}\text{OH}$ radicals reacting with proteins does not lead to their inactivation (reactions with sites distant from the active site). Similar results were obtained, i. a., by Sanner and Phil [2] and Santiard *et al.* [14].

The values of the rate constants for the reaction of hydroxyl radicals with ADH and GAPDH are very high in comparison with other proteins. E.g., the rate constants for the reactions of $^{\circ}\text{OH}$ with haemoglobin, albumin and catalase are $3.6 \cdot 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, $6.9 \cdot 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, and $2.6 \cdot 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, respectively. Since these enzymes play an important role in cellular metabolism, their high reactivity with $^{\circ}\text{OH}$ may lead to serious derangement of cell function and be a significant mechanism of the cellular effects of oxidative stress.

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REFERENCES

1. Buxton, G.V., Greenstock, C. L., Helman, W.P. and Ross, A.B. Critical review of rate constants for reactions of hydrated electrons, Hydrogen atoms and hydroxyl radicals ($^{\circ}\text{OH}/^{\circ}\text{O}^-$) in aqueous solution. **J. Phys.Chem. Ref. Data** 17 (1988) 513-886.
2. Sanner, T. and Pihl, A. Identification of the water radicals in X-ray inactivation of enzymes in solution and determination of their rate of interaction with the enzyme. **Bioch. Biophys. Acta**, 146 (1967) 298-301.
3. Hummel, A. Kinetics in radiation chemistry. in: **Radiation Chemistry: Principles and Applications.**, VCH Publisher, Inc., 1987, 97-136.
4. Bonnichsen, R.K. and Brink, N.G. in: **Methods in Enzymology** (Colowick, S.P. and Kaplan, N.O. Eds), Liver Alcohol Dehydrogenase 1 (1955) 495-496.

5. Amelunxen, R.E. and Carr, D.O. The crystallization and properties of glyceraldehyde-3-phosphate dehydrogenase isolated from rabbit muscle by a simplified procedure. **Biochim. Biophys. Acta** 132 (1967) 256.
6. Von Sonntag, C. The chemical basis of radiation biology. In: **Enzymes** London-New York-Philadelphia, Taylor & Francis Ltd., 1987, 429-457.
7. Klapper, M.H. and Faraggi, M. Application of pulse radiolysis to protein chemistry. **Q. Rev. Biophys.** 12 (1979) 465-519.
8. Schuessler, H. and Herget, A. Oxygen effect in the radiolysis of proteins. I. Lactate dehydrogenase. **Int. J. Radiat. Biol.** 37 (1980) 71-80.
9. Puchała, M. and Schuessler, H. Oxygen effect in the radiolysis of proteins. III. Haemoglobin. **Int. J. Radiat. Biol.** 64 (1993) 149-156.
10. Bielski, B.H.J., Cabelli, D.E. and Arudi, R.L. Reactivity of HO_2/O_2^- radicals in aqueous solution. **J. Phys. Chem. Data** 14 (1985) 1041-1100.
11. Obinger, C., Furtmüller, P.G., Burner, U., Jantschko, W. and Regelsberger, G. Two-electron reduction and one-electron oxidation of organic hydroperoxides by human myeloperoxidase. **FEBS Lett.** 484 (2000) 139-143.
12. Hayon, E., Ibata, T., Lichtin, N.N. and Simic, M. Sites of attack of hydroxyl radicals on amides in aqueous solution. II. The effect of branching to carbonyl and to nitrogen. **J. Am. Chem. Soc.** 93 (1971) 5388-5394.
13. Schuessler, H. Navaratnam, S. and Distel, L. Pulse radiolysis on histones and serum albumin under different ionic conditions. **Rad. Phys. Chem.** 61 (2001) 123-128.
14. Santiar, D., Ribiere, C., Nordman R. and Houee-Levin, C. Inactivation of Cu, Zn-superoxide dismutase by free radicals derived from ethanol metabolism: A γ radiolysis study. **Free Rad. Biol. Med.** 19 (1995) 121-127.