Inactivation of the Na,K-ATPase by radiation-induced free radicals
Evidence for a radical-chain mechanism

K. Hitschke, R. Bühler, H.-J. Apell*, G. Stark*

Department of Biology, University of Konstanz, Postfach 5560, D-78434 Konstanz, Germany

Abstract Free radicals produced by water radiolysis were used to study the inactivation of the enzymatic activity of the Na,K-ATPase. A decrease of the activity to virtually zero with a mono-exponential dependence on the radiation dose was observed. The inactivation process is initiated by hydroxyl radicals. This was shown by the effect of appropriate radical scavengers such as t-butanol, formate and vitamin C. In all cases a significant increase in the characteristic D_{37} dose of inactivation was observed. Inactivation was found to show a so-called inverse dose-rate effect, i.e. the sensitivity of the enzyme to radical attack is increased if the dose rate is reduced. The data were found to agree with the relationship $1/D_{37} \sim 1/\dot{D}^{1/2}$, which is known to be a strong indicator of a radical chain mechanism. This means that the inactivation, after initiation by single radicals, is amplified by a subsequent chain mechanism.

Key words: Na,K-ATPase; Enzymatic activity; Ionizing radiation; Free radical; Inverse dose rate effect

1. Introduction

Free radical species such as the hydroxyl radical OH• or the oxygen radicals O_2^-/HO_2^- are well-known for their deleterious effects and have been discussed in relation with many human diseases [1]. Free radicals are formed throughout normal cellular metabolism. A further source is water radiolysis, i.e. radiation-induced ionization or dissociation of water molecules. The resulting primary or secondary radicals, in view of their free mobility and their reactivity towards cellular components, strongly contribute to the cellular damage produced by ionizing radiation. This indirect radiation effect has to be distinguished from the direct radiation effect produced by immediate absorption of radiation by biological macromolecules. The present communication reports on the effect of free radicals on the Na,K-ATPase, an important enzyme of the plasma membrane of animal cells, which is responsible for the generation of the membrane potential between the cytoplasm and the extracellular medium. Effects of radicals on this ion-transport enzyme have been discussed as a major source of reperfusion damage following ischaemia of the myocardium after myocardial infarction or during long-term coronary bypass procedures [2]. We have found that the inactivation of the enzyme shows a so-called inverse dose rate behavior. This term indicates an increased sensitivity to radical attack of the system under study if smaller dose rates are applied (i.e. at smaller rates of radical production). In the context of radiobiological research, inverse dose rate phenomena are considered as a possible source of an underestimation of cellular radiation damage at small radiation doses. The 'normal' dose rate behavior usually observed in radiobiology shows a decreasing (or constant) effect with decreasing dose rate.

2. Materials and methods

Sodium dodecylsulfate (SDS) was obtained from Pierce Chemical (Rockford, IL). Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, NADH, and ATP (disodium salt; Sonderqualität) were supplied by Boehringer (Mannheim). Sodium cholate and all other reagents (at least analytical grade) were from Merck (Darmstadt).

Na,K-ATPase was prepared from outer medulla of rabbit kidney by procedure C of Jørgensen [3]. The specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay [4] and the protein concentration by the Lowry method [5]. Bovine serum albumin was used as a standard. For all preparations the specific activity was in the range between 1800 and 2300 μmol P_i per h and mg protein at 37°C.

To prepare membrane fragments for the irradiation procedure they were suspended twice in buffer of 10 mM NaCl, pH 5–6, and centrifuged in a Beckman airfuge at 160,000 × g. Under standard conditions the final suspension of fragments was diluted in the same buffer to a concentration of 0.2 μg/μl. Samples of 40 μl were pipetted into tiny polyethylene vessels, of which 9 could be placed in appropriate holes drilled in a square piece of Plexiglas. They were placed at a distance of 6 cm from the focus of an X-ray tube (Philips-Müller RT 100). Attenuation of the 80 kV X-rays was obtained by aluminum filters of various thickness. Underneath the sample an ionization chamber (PTW DL 4; Pychlau, Freiburg) was mounted to control the applied dose. Calibration of the chamber was performed by Fricke dosimetry.

For each series of experiments one control sample was stored at room temperature, while the irradiation procedure started with 9 samples. After appropriate times corresponding to defined doses, samples were successively removed from under the X-ray tube. At the end of the irradiation period, all samples were stored on ice until the specific ATPase activity was determined. For each series of irradiation the decrease of activity was calculated relative to the activity of the control sample in order to compensate for the differences between various protein preparations.

3. Results

The specific activity of Na,K-ATPase-containing membrane fragments was found to decrease to virtually zero as the radiation dose was increased. A typical inactivation curve is shown in Fig. 1. The decay of the activity can be fitted by a single exponential,

$$A(D) = A(0) \cdot \exp(-D/D_{37})$$

where D_{37} is the dose which reduces the initial activity to 37%. Under standard conditions (i.e. with a protein concentration of 0.2 μg/μl (and a dose rate of 75 Gy/min) an average D_{37} value of 200 ± 33 Gy was determined from 27 experiments. No signif-

*Corresponding authors. Fax: (49) (7531) 883 183.

icant difference was found if NaCl was replaced by KCl, which changed the enzyme to a different conformation ($E_1 \rightarrow E_2$) [6].

There was, however, a pronounced dependence of the D_{37} dose on the concentration of membrane fragments in water (Fig. 2). The D_{37} dose showed an increase from 30 Gy to about 900 Gy when the protein concentration was changed from 15 ng/μl to 0.8 μg/μl (Fig. 2A). In a double logarithmic plot, a linear relationship was found between the D_{37} dose and the protein concentration, c_P (slope = 0.81). Thus the ratio, c_P/D_{37}, is fairly constant and shows only a small increase in the range of c_P values applied (Fig. 2B).

The results represented in Fig. 2 show a significant enhancement of the sensitivity of the Na,K-ATPase towards ionizing radiation with increasing dilution of the protein. This may be considered as a strong indication of a so-called indirect radiation effect caused by free radicals of water radiolysis [7]. The phenomenon may be qualitatively explained in the following way: the number of free radicals generated per protein molecule is inversely correlated with the concentration, c_P, of the protein at an identical dose of radiation. Therefore, the rate of radical reactions with the protein, which finally leads to enzyme inactivation, will decrease with increasing c_P. This is in contrast to direct radiation effects (i.e. ionization events at the protein molecules by direct absorption of radiation). In this case the probability of absorption per protein molecule is expected to be independent of the protein concentration.

There are further arguments in favor of a radical-induced inactivation mechanism. (i) In the absence of water or in the frozen state, where free diffusion of the radicals is eliminated, the D_{37} dose is of the order of 5–$10 \cdot 10^4$ Gy, i.e. about two orders of magnitude larger compared to the situation in fluid water. The enzyme is inactivated by the direct radiation effect under these conditions, which have been applied in order to estimate the molar mass of the functional units of the Na,K-ATPase [8,9]. (ii) The D_{37} dose is significantly increased in the presence of various radical scavengers (Table 1). Substances such as vitamin C, t-butanol or formate are known to eliminate or to convert the free radicals of water radiolysis into radicals of lower reactivity (see section 4).
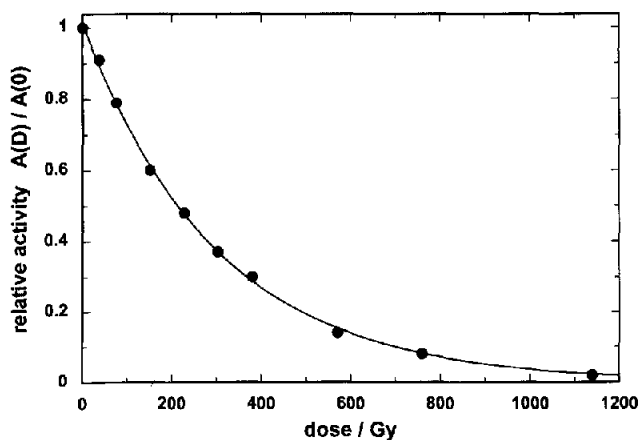

Fig. 1. Inactivation of Na,K-ATPase containing membrane fragments induced by 80 kV X-rays. Membranes with 0.25 μg/μl protein were suspended in an aqueous phase containing 20 mM NaCl and no buffering agents, ~pH 5. The dose rate was 75 Gy/min. The line represents a fit of the function, $A(D) = A(0) \cdot \exp(-D/D_{37})$, to the data with $D_{37} = 307 \pm 4$ Gy (D = dose).
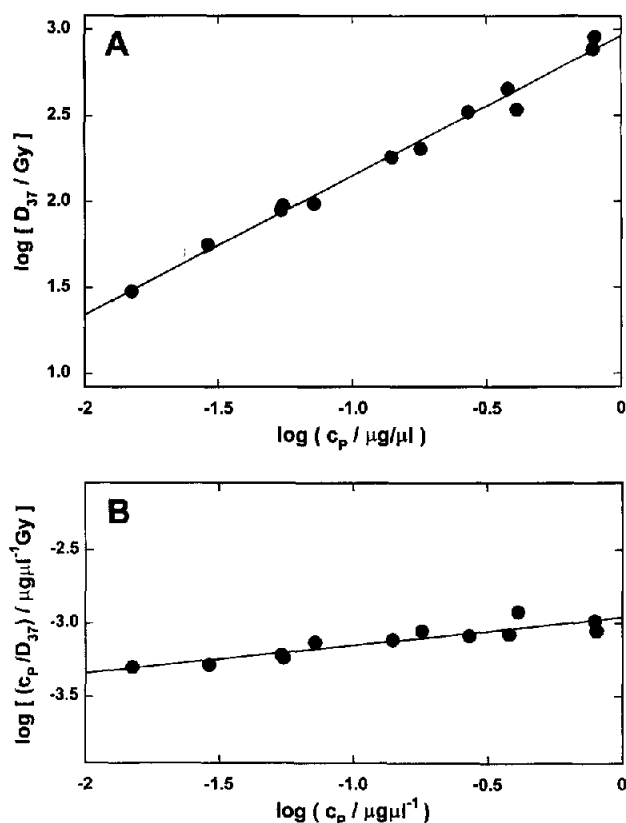

Fig. 2. Inactivation of Na,K-ATPase as a function of the protein concentration, c_P. The experimental conditions apart from the protein concentration agree with those of Fig. 1. (A) A double logarithmic plot of D_{37} vs. c_P shows a linear correlation. (B) Representation of the same data as c_P/D_{37} vs. c_P.

There is a further experimental result which may be explained by the assumption of a radical-induced inactivation process: the D_{37} dose is shifted to smaller values as the dose rate is decreased (Fig. 3). Such a behavior is usually designated as an inverse dose rate effect [10]. The data show a linear relationship between the inverse of the characteristic dose of inactivation, $1/D_{37}$, and the inverse of the square root, $1/\dot{D}^{1/2}$, of the dose rate $\dot{D}$, i.e. $1/D_{37} \sim 1/\dot{D}^{1/2}$ (Fig. 4). A relationship of this kind has been found to hold for radical-chain mechanisms such as radiation- induced lipid peroxidation (see below).

Table 1
Influence of scavengers on the D_{37} dose of Na,K-ATPase inactivation under standard conditions (0.2 μg/μl protein in 10 mM NaCl, pH 5, dose rate 75 Gy/min)

Scavengers		$D_{37}(+) / D_{37}(-)$
Vitamin C	(1 mM)	0.95
Vitamin C	(20 mM)	1.22
Vitamin C	(50 mM)	2.30
t-Butanol	(20 mM)	3.6
t-Butanol	(50 mM)	3.3
Formate	(20 mM)	5.7
Formate	(50 mM)	7.0

The effect is shown as a ratio of D_{37} doses in the presence ($D_{37}(+)$) and in the absence ($D_{37}(-)$) of scavengers. The ratio may be reduced by action of scavenger-derived free radicals (see text).
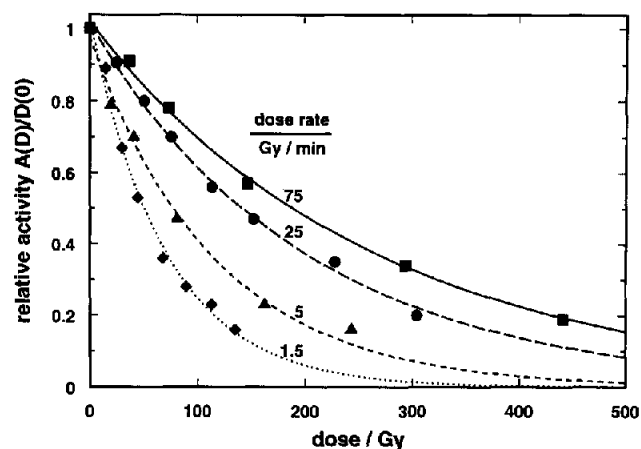
Fig. 3. Dependence of the inactivation of Na,K-ATPase containing membrane fragments on the dose rate. Membrane fragments were irradiated under standard conditions with the indicated dose rates. The decreasing enzymatic activity can be fitted by a single exponential with D_{37} values of 262 Gy (at 75 Gy/min), 191 Gy (at 25 Gy/min), 115 Gy (at 5 Gy/min) and 70 Gy (at 1.5 Gy/min).

4. Discussion

The effect of free radicals on the ion pump Na,K-ATPase has been studied by various groups in recent years [11–17]. All of them used chemically induced radicals by application of Fe^{2+}/H_2O_2, Fe^{2+}/ascorbate or by irradiation of the system with visible light in the presence of photosensitizers. These methods are well known for their efficiency to induce lipid peroxidation [18], which has been suggested to represent an important pathway for the inhibition of the activity of this important enzyme. In spite of previous investigations, the mechanism of inactivation of the Na,K-ATPase by free radicals has remained largely unknown. Even the fundamental question of whether inactivation proceeds indirectly via radical-induced lipid peroxidation and/ or from direct interaction of free radicals with the protein, has not been answered so far.

In the present study free radicals were obtained by water radiolysis. The nature and the concentration of radiation-induced radicals is well known in view of the extensive previous studies in the field of radiation chemistry (cf. [10] or [19] for a review). The primary radicals of water radiolysis are the $OH^\bullet$, the $H^\bullet$, and the $e_{aq}^{\bullet-}$ radicals. In the presence of oxygen, $H^\bullet$ and $e_{aq}^{\bullet-}$ are converted into the secondary superoxide radical $O_2^{\bullet-}$ and into the perhydroxyl radical $HO_2^\bullet$. The two oxygen radicals represent a conjugated acid–base pair. The pK_a of the weak acid $HO_2^\bullet$ is 4.8, so that at pH < 4.8 the perhydroxyl radical and at pH > 4.8 the superoxide radical is predominant.

Air-saturated aqueous solutions, as discussed above, contain a mixture of $OH^\bullet$ and of oxygen radicals $O_2^{\bullet-}/HO_2^\bullet$. The concentration of radicals, c_R, produced by the radiation dose, D, is given by the G_R values of the radicals ($G_R(OH^\bullet) = 2.7$, $G_R(O_2^{\bullet-}/HO_2^\bullet) = 3.2$) according to

$$c_R = 1.03 \cdot 10^{-7} \frac{M}{Gy} \cdot G_R \cdot D \tag{1}$$

The effect of the two kinds of radicals may be distinguished from one another by addition of radical scavengers [10,19]: vitamin C is known to react with both types of radicals present. t-Butanol is a well-known scavenger for $OH^\bullet$ radicals, and

formate converts the $OH^\bullet$ radicals into less reactive $HO_2^\bullet$ radicals. All these substances have been found to increase the D_{37} dose of inactivation (Table 1). This may be understood as clear evidence for a participation of $OH^\bullet$ radicals at the inactivation. Their importance is certainly larger than one may deduce from the values of the ratio $D_{37}(+)/D_{37}(-)$. The radicals generated at the scavenging process may be expected to have a reduced, but non-zero, probability of interaction with the enzyme, i.e. they will also contribute to the inactivation of the enzyme, though at a smaller reaction rate.

Inactivation of the Na,K-ATPase shows the strange phenomenon of an inverse dose rate effect (Fig. 3). This is similar to the inactivation of ion channels formed by the polyene antibiotics amphotericin B and nystatin in planar lipid membranes [20]. An increase in the sensitivity by 2–3 orders of magnitude has been observed in that case at sufficiently small dose rates. The effect has been explained on the basis of a radical chain mechanism well known from lipid peroxidation. A simplified version of the underlying reaction scheme is given as follows:
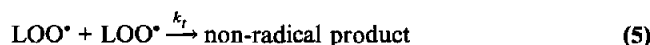
Initiation (by a radiation-induced radical species $X^\bullet$, e.g. $OH^\bullet$):

$$LH + X^\bullet \xrightarrow{k_i} L^\bullet + XH \tag{2}$$

Propagation of the chain:

$$L^\bullet + O_2 \xrightarrow{k_o} LOO^\bullet \tag{3}$$

$$LOO^\bullet + LH \xrightarrow{k_p} LOOH + L^\bullet \tag{4}$$

Termination of the chain:

$$LOO^\bullet + LOO^\bullet \xrightarrow{k_t} \text{non-radical product} \tag{5}$$

Eqs. 2–5 predict an inverse dose rate behavior. This follows from the dose rate dependence of the kinetic chain length, v, defined by

$$v = \frac{\text{number of } O_2 \text{ molecules consumed}}{\text{initiating radical } X^\bullet} \tag{6}$$
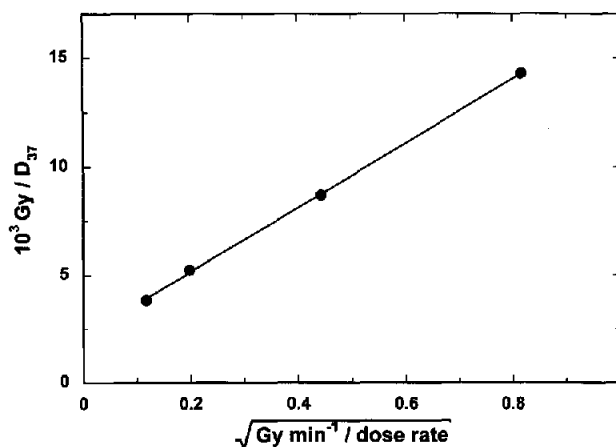

Fig. 4. Inverse dose rate behavior of the inactivation of Na,K-ATPase containing membrane fragments. The data of Fig. 3 show a linear relation between the reciprocal of the D_{37} dose and the reciprocal of the square root of the dose rate. This is indicative of a radical chain mechanism (see text).

v was found to be proportional to the inverse, $1/\dot{D}^{1/2}$, of the square root of the dose rate, $\dot{D}$ [10]. Consequently the radical chain mechanism represented by Eqs. 2–5 shows the same dose rate dependence as was observed previously at the inactivation of polyene channels [20] and is now reported for the inactivation of the Na/K-ATPase (Fig. 3). Application of Eq. 6 requires a linear relationship between the extent of oxidative damage and the inactivation of ion channels or enzyme molecules, respectively.

In the case of polyene channels the occurrence of a free radical-induced chain reaction has been explained on the basis of a peroxidation process of the lipid-like polyene moieties of amphotericin B and nystatin. They are commonly believed to represent the exterior of the ion channels formed by these substances, which are in direct contact with the surrounding lipid phase. Peroxidation of this part of the ion channel has been suggested to destabilize the channel structure, finally leading to an irreversible closure of the open channels [20].

In the case of the Na,K-ATPase the situation is more complex. At least two different kinds of reaction chain mechanisms may be imagined which give rise to the inactivation of the enzyme: inactivation could be caused by products of lipid peroxidation formed according to the (simplified) mechanism outlined above. Free radical-mediated chain mechanisms may, however, also exist without membrane lipids being involved. Neuzil et al. [21] have recently reported that lipid-free protein systems, such as aqueous solutions of bovine serum albumin or lysozyme, show radical-induced chain oxidations. They found that up to 20 amino acids may be modified by a single hydroxyl- or superoxide radical generated by γ-irradiation. For the Na,K-ATPase, discrimination between a protein-based and a lipid-based chain mechanism is difficult, since the enzyme is active only in the presence of a sufficient lipid environment. Experiments with Na,K-ATPase reconstituted in lipid vesicles of different composition (e.g. with a varying degree of unsaturation of the fatty acid residues, experiments under progress) will possibly contribute to a clarification of this question.

There is a twofold aim of the present report: to provide clear evidence of the role of OH· radicals in the inactivation process, and for the first time to demonstrate an inverse dose rate behav-
ior – as an indicator of a radical chain mechanism – for an important enzyme of the plasma membrane.

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