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RADIATION INACTIVATION OF ($Na^+ + K^+$)-ATPase

A SMALL TARGET SIZE FOR THE K^+-OCCLUDING MECHANISM

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Radiation inactivation of partially purified ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) from pig kidney outer medulla shows that the target size for Rb^+ occlusion by the enzyme (in the absence of phosphorylation) is much smaller than the target size for *p*-nitrophenyl phosphatase activity, which is itself smaller than the reported target size for ($Na^+ + K^+$)-ATPase activity.

The combined results of several different experimental approaches [1–5] have led to the hypothesis that the transport of K^+ (or Rb^+) through the ($Na^+ + K^+$)-ATPase system involves a form of the unphosphorylated enzyme in which the transported ions are occluded, so that they are unable to exchange with similar ions in the bathing solutions. The occluded-Rb^+ form of the enzyme can be generated either by Rb^+-catalysed hydrolysis of the phosphoenzyme [1,5], or by combination of Rb^+ with unphosphorylated enzyme [2–4] (see Fig. 1). The latter process is thought to involve only the binding of Rb^+ to low-affinity sites on the E_1 form of the unphosphorylated enzyme, followed by a conformational change from E_1Rb to E_2Rb (see the legend to Fig. 1 for a definition of these terms). We report, here, experiments in which radiation inactivation has been used to determine the apparent target size of the part of the enzyme molecule that is essential for the occlusion of Rb^+ by the route that does not involve dephosphorylation.

The principle of the method was (i) to freeze-dry the partially purified ($Na^+ + K^+$)-ATPase (specific activity 12.4 $\mu mol \cdot mg^{-1} \cdot min^{-1}$), (ii) to irradiate it with high energy electrons, and (iii) to test for Rb^+-occluding ability by suspending the enzyme for 5–10 min at 20°C in a suitable medium containing a low concentration of $^{86}RbCl$, and then forcing the suspension through a K^+-loaded cation-exchange column at a rate that was carefully controlled so that the enzyme spent 0.9 s in contact with the resin. This period was long enough for virtually all of the free Rb^+ to be bound by the resin [3,4], and yet was short compared with the time-constant for the conforma-

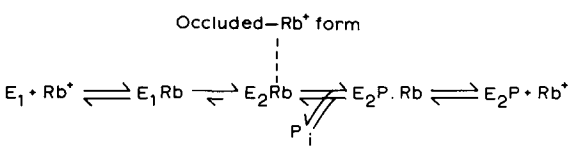

Fig. 1. Scheme (see Refs. 2 and 3) showing the two routes to the occluded-Rb^+ form of the enzyme. E_1 represents the form of the unphosphorylated enzyme, with a low affinity for Rb^+, that is stable in predominantly Na^+ media. E_2Rb represents the form of the unphosphorylated enzyme, with Rb^+ occluded in it, that is stable in Na^+-free media containing Rb^+. P_i represents orthophosphate. E_2P represents the K^+ (or Rb^+)-sensitive form of the phosphoenzyme [16]. No attempt has been made to show the stoichiometry.

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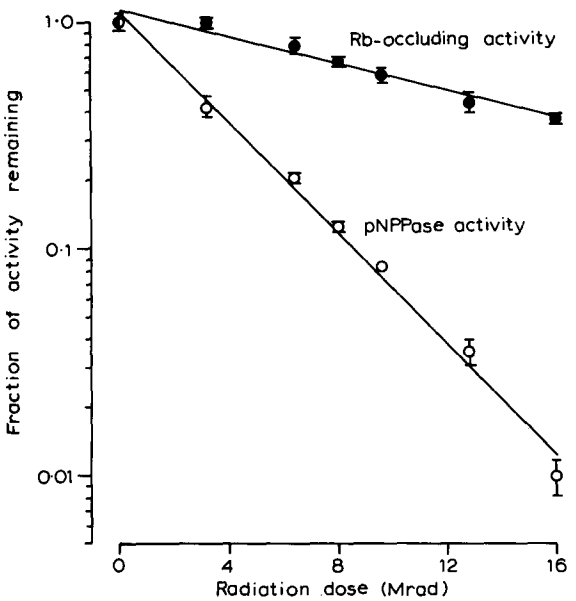

Fig. 2. The effect of increasing doses of radiation on the ability of $(Na^+ + K^+)$-ATPase (a) to occlude Rb^+, and (b) to catalyse the K^+-dependent hydrolysis of *p*-nitrophenyl phosphate. $(Na^+ + K^+)$-ATPase (specific activity 12.4 μmol · mg^{-1} · min^{-1}) prepared from pig kidney outer medulla [15] was suspended at a concentration or 1.6 mg/ml in a medium containing 25 mM histidine (pH 7.4 at 20°C), 1 mM EDTA (Tris salt) and 250 mM sucrose. Samples of the suspension, 200 μl in volume, were lyophilized in glass tubes overnight, and the lyophilized samples were irradiated with high-energy electrons using a clinical MEL SL75/20 20 MeV linear accelerator, at a dose rate of 2.5 Mrad · min^{-1} as described by Ellory et al. [17]. During irradiation, the sample temperature was in the range 10–40°C, and control experiments showed that the exposure of lyophilized microsomes to temperatures in this range did not affect their $(Na^+ + K^+)$-ATPase activity. After the irradiation, 0.1 ml of water was added to each tube, and the enzyme was suspended by gentle vortex mixing and assayed (a) for its ability to occlude Rb^+, and (b) for its K^+-dependent *p*-nitrophenyl phosphatase activity. To measure the ability of the enzyme to occlude Rb^+, 70 μg portions of enzyme protein were suspended in 0.5 ml portions of a medium containing 100 μM ^{86}RbCl, 100 mM Tris/Tris-HCl (pH 7.4 at 20°C), 0.5 mM EDTA (Tris salt), without or with 15 mM NaCl, and the suspensions were passed down K^+-loaded columns of Dowex-50W X 8 sulphonic resin (100–200 mesh), at 20°C at a rate such that the enzyme spent 0.9 s in contact with the resin. Effluents were assayed for radioactivity and protein [3]. Since virtually all of the occluded Rb^+ is released in the presence of 15 mM NaCl (unpublished work by Richards, D.E. and Glynn, I.M.), the extra radioactivity carried through the columns when NaCl was absent from the suspending medium gave a measure of the ability of the enzyme to occlude Rb^+. Each point in the Figure represents the difference (±S.E.) between the means of four determinations with no NaCl in the suspending medium and four determinations with 15 mM NaCl in the suspending medium. The amount of Rb^+ occluded by the unirradiated enzyme in this experiment was 1.41 nmol per mg protein. The *p*-nitrophenyl phosphatase activity was assayed in media containing 10 mM *p*-nitrophenyl phosphate (diTris salt), 20 mM $MgCl_2$ and either 100 mM KCl or 100 mM choline chloride. The temperature was 37°C and the pH 7.2. The reaction was stopped by the addition of four volumes of 0.1 M NaOH, and the free *p*-nitrophenol was assayed spectrophotometrically at 412 nm. Because the range of activities in the different samples was so wide, both the concentration of enzyme and the duration of the incubation were varied over a 20-fold range so as to give convenient final concentrations of *p*-nitrophenol. Assays were done in triplicate, with two incubation periods for each. The K^+-dependent *p*-nitrophenyl phosphatase activity of the unirradiated enzyme (2.6 μmol · mg^{-1} · min^{-1}) accounted for over 98% of the total *p*-nitrophenyl phosphatase activity, but this fraction had fallen to 65% in the most heavily irradiated sample. (The cause of this fall could be either that the small amount of K^+-independent *p*-nitrophenyl phosphatase activity is less sensitive to irradiation, or that irradiation can eliminate the K^+-dependence of the K^+-dependent *p*-nitrophenyl phosphatase without totally abolishing its hydrolytic activity.) pPNPPase, *p*-nitrophenyl phosphatase.

tional change (E_2Rb to E_1Rb) that precedes the release of Rb^+ from the enzyme, about 5 s at 20°C [2,4,6,7]. Under these conditions 84% of the occluded Rb^+ would be expected to emerge with the enzyme at the bottom of the column. To provide an internal standard, the irradiated samples were also tested for K^+-dependent *p*-nitrophenyl phosphatase activity. This activity was chosen rather than the $(Na^+ + K^+)$-ATPase activity, simply because it is so easily measured. Experimental details are given in the legend to Fig. 2.

The results of one of three experiments are summarised in Fig. 2. The logarithm of the *p*-nitrophenyl phosphatase activity appears to be linearly related to the radiation dose, and the dose required to reduce the activity to $1/e$ of its original value (D_{37}) is 3.9 Mrad. Assuming 'single-hit inactivation' [9], and using the equation:

$$\text{target size} = (6.4 \cdot 10^5)/(D_{37} \text{ in Mrad}),$$

derived both from theoretical and empirical work [8–10], we calculate a target size for K^+-dependent *p*-nitrophenyl phosphatase activity of 164 000. This is

comparable with the figures of 140000 and 180000 for the target sizes of the K^+-dependent *p*-nitrophenyl phosphatase activitires of guinea-pig kidney microsomes [8] and of red cell membranes [17], respectively. The occlusion measurements show some scatter, but the slope of the line obtained by plotting the logarithm of Rb^+ occlusion against radiation dose corresponds to a target size of about 39000. In two further, similar, experiments, the slopes gave target sizes of about 42000 and about 60000. All of these figures are much smaller than estimates of the target size for the K^+-dependent *p*-nitrophenyl phosphatase activity (see above), which are themselves smaller than estimates of the target size for $(Na^+ + K^+)$-ATPase activity (190000–300000 [8], 330000 [17], 327000 [12]).

The low figures for the target size for Rb^+ occlusion, taken at their face value, imply that the ability to occlude Rb^+ resides in a relatively small part of the whole enzyme. The enzyme molecule is thought to contain two copies of a large peptide with an M_r of about 120000 [11,12] and two copies of a small glycopeptide of which the protein portion has an M_r thought to be in the region 40000–60000 [11,12]. At first sight, it is tempting to suppose that the small target size for Rb^+ occlusion corresponds to the M_r of the glycopeptide. There are, however, reasons for caution. In the first place, the assumption of one-hit-inactivation may be wrong, since the measurements of Rb^+ occlusion are not sufficiently accurate for us to be certain that the relation between the logarithm of the residual activity and the radiation dose is strictly linear. Secondly, even if the estimates of the target size for Rb^+ occlusion are roughly correct, we cannot rule out the possibility that the target is a functionally distinct part of the large peptide. It is true that, in most radiation-inactivation studies, when target sizes do not correspond to the molecular weight of the whole enzyme they turn out to be equivalent to the weight of a subunit [13]. The great majority of the enzymes that have been studied in this way, however, are soluble enzyme. We know that part of the large peptide is intimately associated with lipid [14], and it is conceivable that the lipid can restrict the transfer of energy following the primary ionisation sufficiently to allow part of the damaged peptide to continue to function. What is not in doubt is that the enzyme as a whole can retain its ability to occlude Rb^+ even when it has suffered radiation damage sufficient to prevent both $(Na^+ + K^+)$-ATPase activity and K^+-dependent *p*-nitrophenyl phosphatase activity.

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