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Oligomycin interaction with Na,K-ATPase: oligomycin binding and dissociation are slow processes

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Oligomycin interacts with the Na,K-ATPase by increasing the apparent Na^+ affinity in the non-phosphorylated state of the enzyme. This property is used to estimate rate constants attributed to oligomycin binding and dissociation reactions with Na,K-ATPase. The rate constants are determined indirectly, employing stop-flow fluorimetry of eosin, the fluorescence of which is a marker for the E_1 state of the enzyme, i.e. for Na^+ binding. The second-order rate constants derived for oligomycin binding are in the range (6–12) · 10^4 $M^{-1}s^{-1}$ at 6°C for both shark rectal gland and pig kidney enzyme. Rate constants for dissociation of the enzyme-oligomycin complex are about 0.05 s^{-1} at 6°C. The slow rates of binding and dissociation suggest that oligomycin acts from within the membrane lipid phase rather than from the aqueous phase. The dissociation constant at 6°C for the enzyme-oligomycin complex can be calculated to be about 1 μM for shark enzyme and about 2 μM for kidney enzyme, at pH 7.0 in 2 mM NaCl.

Introduction

The Na,K-ATPase is a large integral membrane-bound cation transport enzyme, responsible for the active transport of Na^+ and K^+ across the plasma cell membrane (see Ref. 1 for a recent collection of reviews). The kinetics of the coupling between the cation transport and hydrolysis of ATP has proved to be very complex, involving several phosphorylated and non-phosphorylated intermediates [2,3]. The major protein conformations in the non-phosphorylated state are denoted E_1 (predominant in Na^+-containing media, binds ATP, ADP and the dye eosin with high affinity) and E_2 (the protein conformation with low affinity for the nucleotides and eosin). The E_2 form has at least two different states, one with K^+ bound (the 'occluded' $E_2(K)$ form, which has a slow rate of transition to the E_1 form), and another E_2 form with no cation occluded. The latter has a relatively rapid rate of transition to E_1 when Na^+ is added [4]. Several other 'sub'-states of the protein can also be determined (discussed in Ref. 5).

The antibiotic oligomycin, an inhibitor of the Na,K-ATPase [6], has been used extensively to characterize the phosphorylated intermediates of the enzyme, the apparent action under turn-over conditions being to block the enzyme in the phosphorylated E_1 state (Ref. 7, also reviewed in Ref. 2). Oligomycin also has the property of inducing occlusion of Na^+ in the non-phosphorylated E_1 state [8], a property recently used in a study of cation-binding site location in the protein [9].

Direct measurements of the properties of oligomycin binding to the enzyme have, however, not been published.

The present paper presents a method to determine kinetic properties of oligomycin binding to the non-phosphorylated state of the enzyme. The fluorescence of eosin – which is high when bound to the E_1 form and low when not bound [10] – is used to estimate the fraction of enzyme in the E_1 form. Since oligomycin increases the Na^+-affinity, a change in the oligomycin concentration will change the fraction of enzyme in the E_1 form at a given Na^+-concentration. Stop-flow measurements of eosin fluorescence can thus be used to monitor the kinetics of the oligomycin interaction with the Na,K-ATPase. Since it is observed that transient changes induced by oligomycin are very slow compared to the other processes involved (Na^+- and eosin-binding), the rate of eosin fluorescence changes can be interpreted in a simple model for oligomycin binding to and dissociation from the enzyme.

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Methods and Materials

Preparation of pig kidney enzyme

Na,K-ATPase was isolated in the membrane bound form from pig kidney outer medulla by the method of Jørgensen [11] followed by selective extraction of the plasma membranes with SDS in the presence of ATP. The enzyme was stored at −20°C in 250 mM sucrose, 12.9 mM imidazole, and 0.625 mM EDTA at pH 7.5. The specific ouabain-inhibitable Na,K-ATPase activity was about 1500 μmol/mg protein per h at 37°C.

Preparation of shark rectal gland enzyme

Na,K-ATPase from the rectal gland from *Squalus acanthias* was prepared as described by Skou and Esmann [12], but without the treatment with saponin. The Na,K-ATPase typically constituted 50–70% of the protein (determined as the content of α- and β-subunits from SDS-gel electrophoresis), and the specific activity was about 1500 μmol/mg protein per h. Na,K-ATPase activity and protein content was determined as previously described [13].

Equilibrium fluorescence measurements

The fluorescence of eosin as a function of the Na^+ concentration (see Fig. 1) was measured at 23°C in a Perkin-Elmer MPF 44A spectrofluorimeter. Excitation was at 530 nm, and emission was monitored at 560 nm (both slits being 10 nm). The sample contained 0.025 mg shark Na,K-ATPase per ml in 30 mM histidine (pH 7.0), 1 μM eosin and 0 or 1 μM oligomycin. NaCl was added to give the indicated final concentrations.

Stop-flow fluorimetry

Measurements of rates of changes in fluorescence were carried out with a SFM-2 stop-flow apparatus (Biologics, France). Excitation was at 530 nm, and emission was measured with a photomultiplier with a cut-off filter at 550 nm. Data were collected with an A/D-converter interfaced to an HP 9816 microcomputer. The signal-to-noise ratio was increased by digitally adding 3–5 tracings. Non-linear least-squares calculation of exponential decays were performed using a programme kindly provided by Robert Clegg, Göttingen.

Samples were prepared in the following way: Both stop-flow syringes contained 30 mM histidine (pH 7.0), 2 mM NaCl and 1 μM eosin. One syringe contained 0.05 mg protein/ml, and the other syringe contained additional NaCl or oligomycin. Oligomycin was added as an ethanol solution, and it was ensured that the final ethanol concentration did not exceed 0.5%. The volume delivered from each syringe was 150 μl per shot, the flow-time being 200 ms. This gives a dead time of about 2 ms in this apparatus, which is sufficiently short for the reactions to be followed in these experiments.

Materials

Oligomycin (M_r 790) was obtained from Boehringer-Mannheim, and eosin was obtained from Koch-Light.

Results and Discussion

The basis for the experiments and results reported in the present paper is shown in Fig. 1. The fluorescence of eosin is given as a function of the Na^+ concentration in the presence of 0.025 mg shark Na,K-ATPase per ml. In the absence of oligomycin the Na^+ concentration required to give half-maximal saturation of the fluorescence is about 2 mM (pH 7.0 at 23°C). It is clear that the addition of 1 μM oligomycin decreases the Na^+ concentration required to give a half-maximal increase in the fluorescence of eosin to less than 1 mM. The maximal fluorescence response is about the same with and without oligomycin, suggesting that the affinity for eosin is about the same in the native and the oligomycin-bound enzyme. It thus appears as if oligomycin simply increases the affinity for Na^+.

In a very simple model (Fig. 2) this can be interpreted as follows: oligomycin binding increases the concentration of enzyme with Na^+ bound, and this leads – at a fixed eosin concentration – to an increase in the concentration of enzyme with eosin bound, and thus to a higher fluorescence (the titration curves are not simple hyperbolic curves, indicating that binding of more than one Na^+ is required for eosin to bind: we will not deal with this aspect in the present paper).

The rate of fluorescence change is very rapid when Na,K-ATPase at a low Na^+ concentration is exposed to a higher Na^+ concentration, Fig. 3 (note that there is no

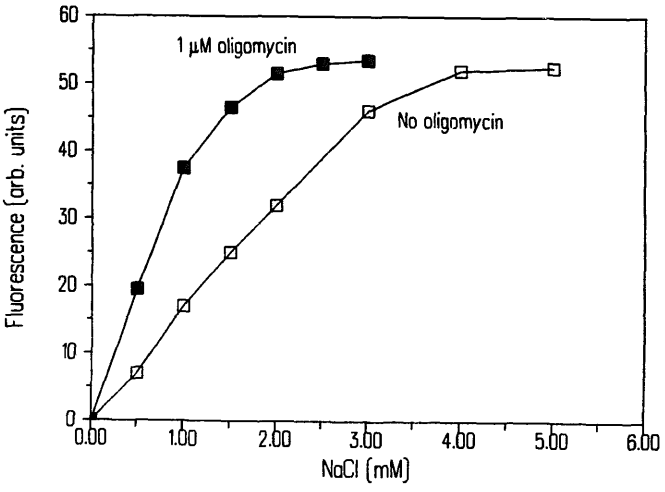

Fig. 1. Titration of eosin fluorescence with Na^+. Shark Na,K-ATPase (0.025 mg/ml) in 30 mM histidine (pH 7.0) and 1 μM eosin is titrated with NaCl in the absence (open symbols) and presence of 1 μM oligomycin (filled symbols). The fluorescence is given in arbitrary units as a function of the NaCl concentration. The experiment is carried out at 23°C.

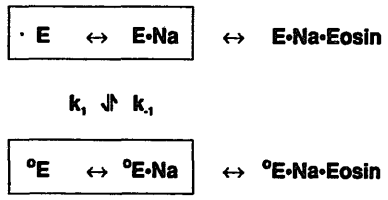

Fig. 2. A simple model for the minimal number of enzyme species involved in Na^+, eosin and oligomycin binding. It is assumed that the binding of oligomycin is either to the empty E form or to the E·Na form (this can not be distinguished here). Transition from the E and E·Na forms to E·Na·eosin form is rapid, cf. Fig. 3.

oligomycin present in the experiments shown in Fig. 3). This indicates a rapid transformation from enzyme with no Na^+ bound (E with no cation occluded) to the E · Na · eosin form, both with shark and kidney enzyme (Figs. 3A and 3B, respectively). In terms of the model in Fig. 2 (upper part), reactions in the horizontal direction are rapid, with halftimes for the fluorescence change being about 300 ms for shark enzyme and 60–80 ms for kidney enzyme (at 6°C). Fig. 3 also shows that the rate of fluorescence change increases when the Na^+ concentration is increased, both for shark and kidney Na,K-ATPase, suggesting that Na^+ bind to the enzyme before the E_1 state is reached, i.e. binds to E_2 (for simplicity the non-liganded E_2 and E_1 forms are abbreviated E in the scheme depicted in Fig. 2; see Refs. 2,4 and 14 for a more detailed discussion of this). It is clear from Fig. 3 that kidney enzyme responds more quickly to a change in the Na^+ concentration than shark enzyme, indicating a more rapid transition from the E_2 to the E_1 state.

If oligomycin binding is slow – relative to the halftimes observed when the Na^+ is increased – it should be expected that the rate of fluorescence increase arising from addition of oligomycin to enzyme at a suboptimal Na^+-concentration (here 2 mM) will reflect the binding reaction of oligomycin.

Fig. 4 shows the fluorescence change when enzyme in 2 mM NaCl, 30 mM histidine and 1 μM eosin is mixed with oligomycin (in the same buffer) at increasing concentrations, i.e. the only change in the medium is an oligomycin concentration-jump (and a 2-fold dilution of the enzyme). Note that the tracings in Fig. 4 are displaced vertically. It is clear that the rate of fluorescence increase is very much slower when the enzyme is pulled towards the E_1 · Na · eosin form using oligomycin than by an increase in the Na^+ concentration (compare Figs. 3 and 4).

Fig. 4 also shows that an increase in the concentration of oligomycin increases the rate of the fluorescence change, both for shark and kidney Na,K-ATPase (Figs. 4A and 4B, respectively. Note that all the transients are normalized to the same amplitude to ease comparison of the transition rates).

The relative amplitude of the fluorescence response of the transients shown in Fig. 4 also depend on the oligomycin concentration, Fig. 5. This figure shows (in arbitrary units) that the amplitude of the fluorescence signal saturates at an oligomycin concentration of about 2 μM. Half maximum fluorescence change is observed at a concentration of about 0.5–0.7 μM oligomycin.

The transients in Fig. 4 were fitted by single exponentials. Fig. 6 shows the dependence of the observed

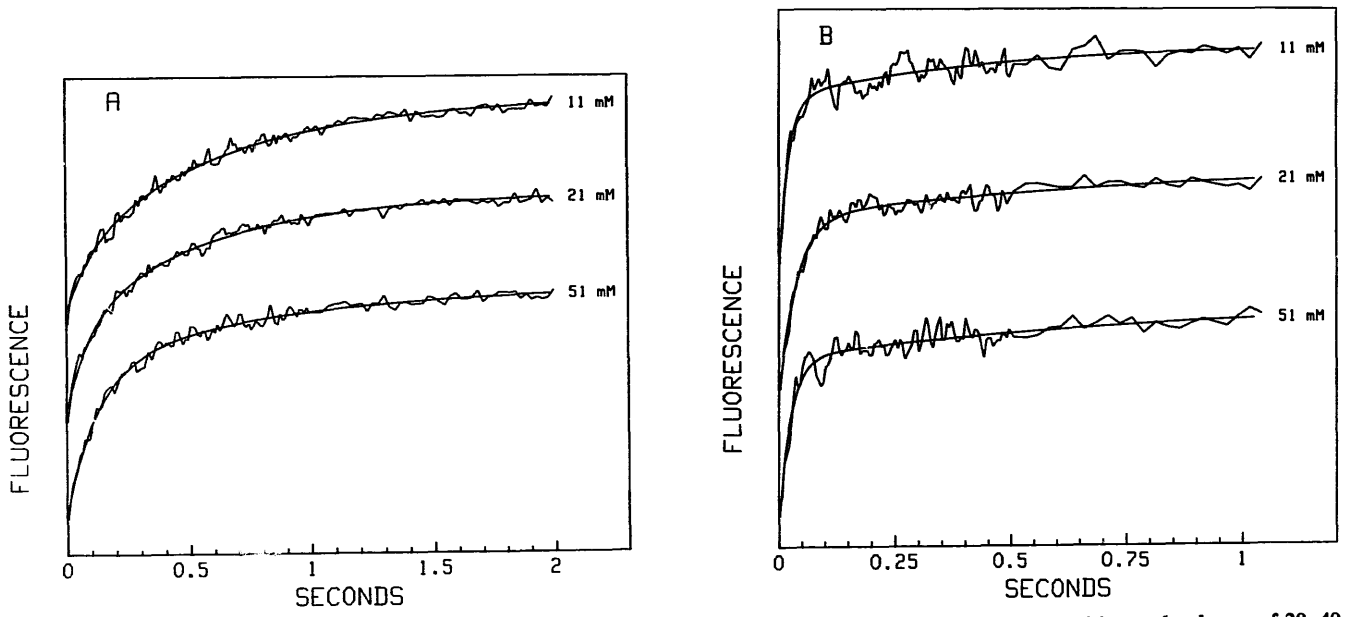

Fig. 3. The effect of a jump in Na^+ concentration on eosin fluorescence. Na,K-ATPase in 2 mM NaCl was mixed with equal volumes of 20, 40 or 100 mM NaCl to give the indicated final concentrations of NaCl. Panel (A) shows experiments with shark enzyme, and panel (B) shows experiments with kidney enzyme. The buffer was 30 mM histidine (pH 7.0 at 6°C) and final concentrations were 0.025 mg protein/ml and 1 μM eosin. The temperature was 6°C.

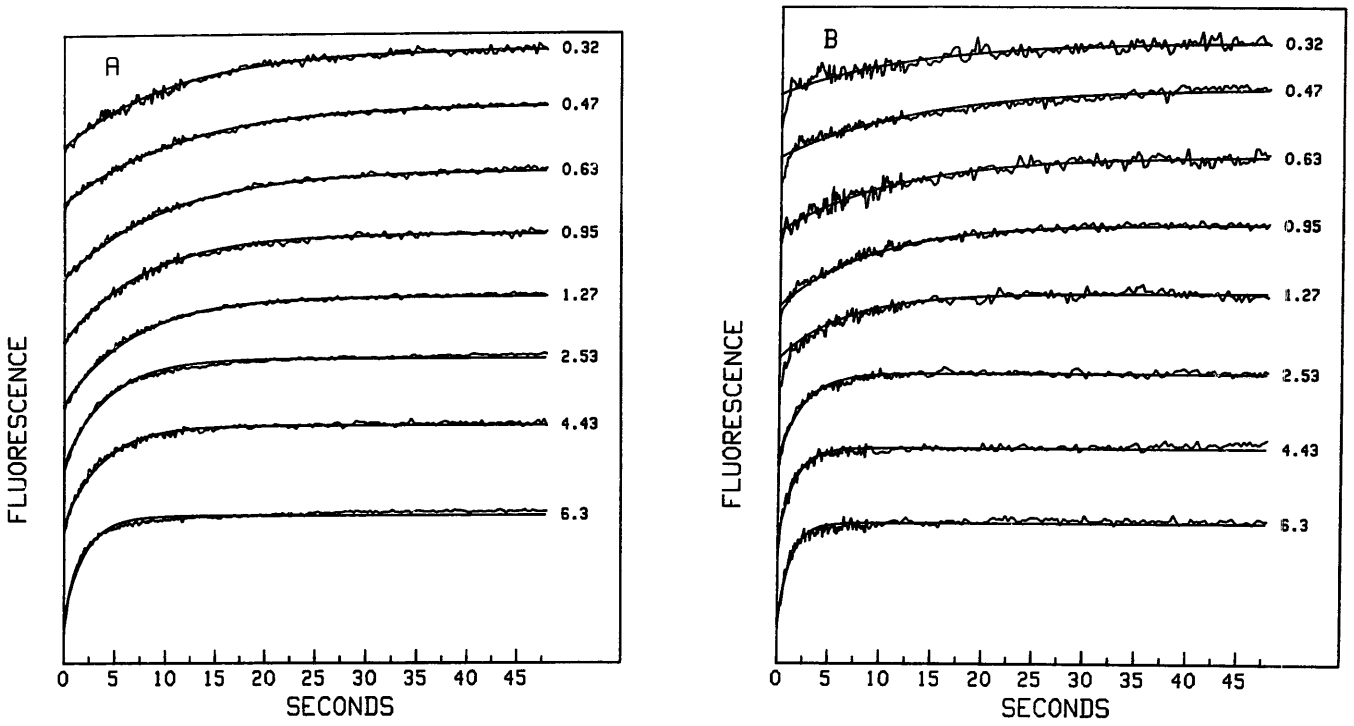

Fig. 4. The effect of a jump in oligomycin concentration on eosin fluorescence. Na,K-ATPase in 2 mM NaCl was mixed with equal volumes of buffer containing 2 mM NaCl and oligomycin to give the indicated final concentrations of oligomycin (in μM). Panel (A) shows experiments with shark enzyme, and panel (B) shows experiments with kidney enzyme. The buffer was 30 mM histidine (pH 7.0 at 6°C) and final concentrations were 0.025 mg protein/ml and 1 μM eosin. The temperature was 6°C. The solid lines represent single exponentials, the rate constants of which are shown in Fig. 6 for each oligomycin concentration. Note that all tracings have been normalized to the same amplitude, and they are displaced vertically to ease comparison. The relative amplitudes are given in Fig. 5.

rate constant on the oligomycin concentration. For both shark and kidney enzyme a fairly linear relationship is observed up to 6.3 μM oligomycin (total concentration after mixing).

The results described in Fig. 6 suggests that the effect of oligomycin (O) can be interpreted as a simple binding/dissociation reaction:

$$E + O \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EO \qquad (1)$$

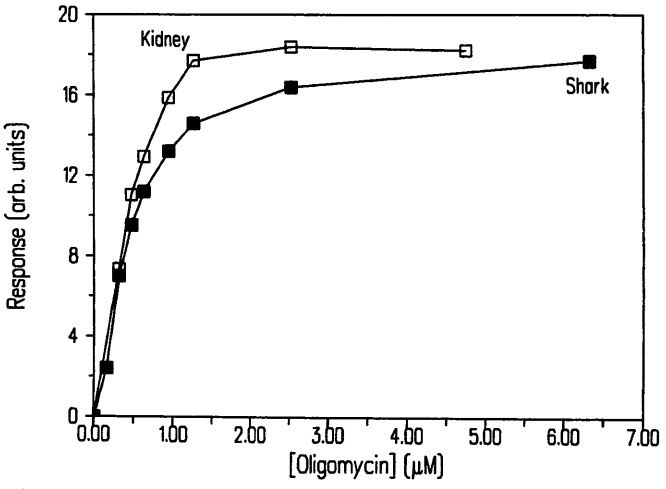

Fig. 5. Amplitude of the fluorescence change when the oligomycin concentration is increased. This figure shows the amplitude of the fluorescence response when Na,K-ATPase in 2 mM NaCl is exposed to increasing concentrations of oligomycin (see Fig. 4). The filled symbols represent shark enzyme, and the open symbols represent kidney enzyme.

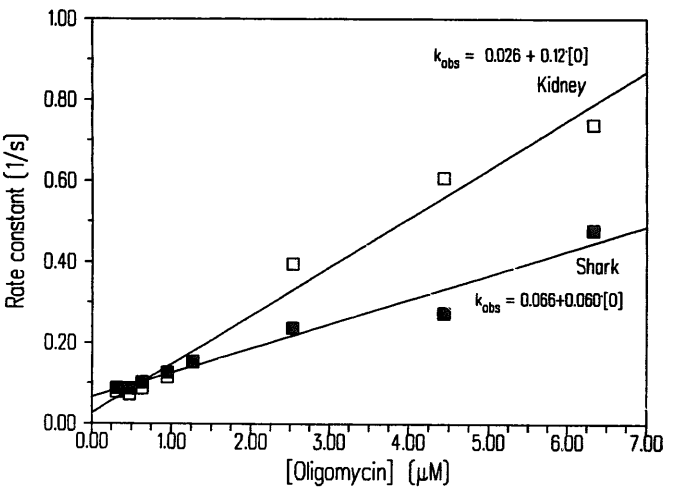

Fig. 6. Rate of fluorescence increase upon a oligomycin concentration jump. The rate constant used to fit the transient fluorescence changes (Fig. 4) is given as a function of the oligomycin concentration. The filled symbols are for experiments with shark Na,K-ATPase, and the open symbols are for experiments with kidney Na,K-ATPase. The straight lines are obtained by regression analysis.

where the affinity for Na^+ is higher for the oligomycin-bound form (EO) than for native enzyme (E). At a given Na^+ concentration, the fraction of enzyme with Na^+ bound – and thus with eosin bound – will increase upon oligomycin binding.

Relaxation of this system (Eqn. 1) upon a change in the oligomycin concentration is a single exponential with a rate constant (k_{obs}), which is related to the oligomycin-concentration in the following way:

$$k_{obs} = k_{-1} + k_1 \cdot [O]$$

where k_{-1} has the dimension s^{-1} and k_1 the dimension $M^{-1}s^{-1}$. The dissociation constant for oligomycin is then k_{-1}/k_1 (M).

Table I summarizes the result of this analysis. The on-rate constant (k_1) is in the order 10^5 $M^{-1}s^{-1}$, which is very small compared to for example the on-rate constant for eosin binding to the same enzyme ($(1–2) \cdot 10^7$ $M^{-1}s^{-1}$, Ref. 14). The dissociation rate constant is also very small, in the range 0.02–0.06 s^{-1} at 6°C

The calculated values for the dissociation constant for the enzyme-oligomycin complex are 1 to 2 μM at 6°C. This is in reasonably good agreement with the half-maximal effect of oligomycin on the fluorescence response, where values slightly less than 1 μM are obtained (Fig. 5). Investigations by Plesner and Plesner also yield values in the micromolar range (at 37°C, Ref. 15).

The present experiments do not tell whether the rate constant derived is for oligomycin binding to enzyme with or without Na^+ bound (i.e. to E or $E \cdot Na$), see Fig. 2, and the increase in Na^+ affinity upon transition from the native enzyme to the enzyme with oligomycin bound can thus not be calculated. Fig. 1 suggests that the apparent affinity for Na^+ is increased 2-fold by 1 μM oligomycin. Increasing the oligomycin concentration to for example 10 μM does not shift the titration curve appreciably to the left (data not shown), suggesting that the effect of oligomycin on the apparent affinity for Na^+ has saturated at 1–2 μM.

The rate constant for de-occlusion of Na^+ from shark enzyme with oligomycin bound is low (about 1 s^{-1} at 23°C) compared to the rate in the absence of oligomycin [8]. In terms of the simple scheme shown in Fig. 2, this implies that reactions within the lower box are slow both for occlusion and de-occlusion (i.e. binding and release) of Na^+, since the increase in Na^+ affinity at limiting oligomycin concentrations is only about a factor 3. The scheme shown in Fig. 2 is a minimal model describing the present results. The two findings, that the rate of transition to the high-fluorescence form is proportional to the oligomycin concentration, and that the magnitude of the fluorescence response saturates at about 1 μM oligomycin, suggest that we are dealing with a simple, but slow binding/dissociation reaction for oligomycin. If the oligomycin reactions were rapid, but followed by a slow occlusion/de-occlusion step involving Na^+, it would be expected that the rate of fluorescence increase would saturate at the same oligomycin concentration which saturates the magnitude of the fluorescence response. This possibility is not compatible with the present data, Figs. 5 and 6 (a more detailed study of the interaction between Na^+, oligomycin and the Na,K-ATPase is in progress).

A possible explanation for the slow rates of binding and release of oligomycin could be that they are not diffusion-controlled aqueous processes, but rather diffusion of oligomycin within the lipid bilayer. In the following, the amount of oligomycin in the membrane phase will be estimated. Although no data are available under the exact conditions of these experiments, a rough estimate will give an idea of the relation between the amount of oligomycin in the water and the membrane phase.

The solubility of oligomycin (data from Ref. 16) in water is about 0.02 mg/ml, and – taking an organic solvent such as ether or ethanol (oligomycin solubility about 300 mg/ml) as representative for the solubility in membrane lipids – it can be calculated that the solubility is a factor 15000 higher in the organic phase. Here is used 0.025 mg protein/ml, which with a lipid/protein weight ratio of about 0.8 [17] gives a lipid 'volume' of about 20 nl/ml, a lipid:water volume ratio of 1:50000. It can thus be calculated that about 30% of the oligomycin actually resides in the lipid-phase, the amount being proportional to the total oligomycin concentration (up to 0.02 mg/ml, the solubility in water). The lipid/oligomycin molar ratio in the membrane can be calculated from the above values to be about 100 at for example 1 μM total oligomycin. This indicates that no gross perturbation of the membrane is to be expected. The molar ratio between oligomycin (residing in the membrane) and Na,K-ATPase phosphorylation sites is about 6 at 1 μM total oligomycin, indicating an excess of oligomycin over enzyme in these experiments (not less than 0.3 μM oligomycin are used in the present experiments). If oligomycin partitions into the bilayer at the lipid/water interface – and is not evenly distributed

TABLE I

Kinetic parameters describing oligomycin binding and release at 2 mM NaCl and 1 μM eosin in 30 mM histidine, pH 7.0 at 6°C

Values are calculated from the data given in Fig. 6, using Eqn. 1. The total oligomycin concentration is here given in mol/l, using a molecular weight of 790.

	Shark	Kidney
Binding rate constant (k_1, $M^{-1}s^{-1}$)	$6.0 \cdot 10^4$	$12 \cdot 10^4$
Dissociation rate constant (k_1, s^{-1})	0.066	0.026
Dissociation constants (μM)	1.1	2.2

within the bilayer – the number of oligomycin molecules per enzyme molecule will of course be smaller.

Conclusion

The present experiments suggest that oligomycin reacts with the Na,K-ATPase in the non-phosphorylated state by effectively increasing the affinity for Na^+. The data are interpreted in a model suggesting very slow rates of oligomycin binding and dissociation, and it is proposed that these processes are taking place not in aqueous solution, but rather within the membrane phase. The dissociation constant for the Na,K-ATPase-oligomycin complex in 2 mM NaCl is about 1 μM, showing that oligomycin is a more potent ligand in the non-phosphorylated enzyme than under turnover conditions, where higher concentrations of oligomycin are required to obtain inhibition of Na,K-ATPase activity [7,15].

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