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Properties of oligomycin-induced occlusion of Na^+ by detergent-solubilized Na,K-ATPase from pig kidney or shark rectal gland

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Oligomycin induces occlusion of Na^+ in membrane-bound Na,K-ATPase. Here it is shown that Na,K-ATPase from pig kidney or shark rectal gland solubilized in the nonionic detergent $C_{12}E_8$ is capable of occluding Na^+ in the presence of oligomycin. The apparent affinity for Na^+ is reduced for both enzymes upon solubilization, and there is an increase in the sigmoidicity of binding curves, which indicates a change in the cooperativity between the occluded ions. A high detergent/protein ratio leads to a decreased occlusion capacity. De-occlusion of Na^+ by addition of K^+ is slow for solubilized Na,K-ATPase, with a rate constant of about 0.1 s^{-1} at 6°C. Stopped-flow fluorescence experiments with 6-carboxyeosin, which can be used to monitor the E_1Na-form in detergent solution, show that the K^+-induced de-occlusion of Na^+ correlates well with the fluorescence decrease which follows the transition from the E_1Na-form to the E_2-form. There is a marked increase in the rate of fluorescence change at high detergent/protein ratios, indicating that the properties of solubilized enzyme are subject to modification by detergent in other respects than mere solubilization of the membrane-bound enzyme. The temperature dependence of the rate of de-occlusion in the range 2°C to 12°C is changed slightly upon solubilization, with activation energies in the range 20–23 kcal/mol for membrane-bound enzyme, increasing to 26–30 kcal/mol for solubilized enzyme. Titrations of the rate of transition from E_1Na to E_2K with oligomycin can be interpreted in a model with oligomycin having an apparent dissociation constant of about 2.5 μM for $C_{12}E_8$-solubilized shark Na,K-ATPase and 0.2 μM for solubilized pig kidney Na,K-ATPase.

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 cell membrane (see Ref. 1 for a recent collection of reviews). The transport of the ions is thought to occur through a set of conformational changes in the protein and in some of the conformations, the so-called 'occluded' states of the enzyme [2], the ions are rendered inaccessible to exchange with ions in solution. Both K^+ and Na^+ can be trapped in occluded states. K^+ (or its congener Rb^+) is spontaneously occluded by the Na,K-ATPase [3,4], whereas occlusion of Na^+ requires either that the enzyme is phosphorylated and halted in the E_1P-state [5,6], or that the antibiotic oligomycin is present [7]. The nature of the occlusion-sites has attracted considerable attention recently, notably with the recent demonstration by Karlish et al. [8] that membrane fragments containing a 19 kDa tryptic fragment of kidney Na,K-ATPase are capable of occluding the cations. It has previously been shown that enzyme from shark rectal gland, solubilized in the nonionic detergent octaethyleneglycol dodecyl monoether ($C_{12}E_8$), can occlude Rb^+ in much the same manner as the membrane-bound Na,K-ATPase [9]. Soluble kidney Na,K-ATPase can also occlude Rb^+ and Na^+, the latter when the enzyme is inhibited and phosphorylated with CrATP [6].

The purpose of the present paper is to investigate the oligomycin-induced occlusion of Na^+ for both shark and kidney Na,K-ATPase with special emphasis on the properties of solubilized enzymes. This involves determination of occlusion capacities, rates of de-occlusion

Abbreviations: $C_{12}E_8$, octaethyleneglycol dodecyl monoether; E_1, the Na,K-ATPase conformation predominant in NaCl; E_2, the Na,K-ATPase conformation predominant in KCl; 6-CEo, 6-carboxyeosin; CDTA, *trans*-1,2-cyclohexylenedinitrilotetraacetic acid.

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and correlation of the de-occlusion rates with fluorescence measurements of conformational changes.

The main result is that the de-occlusion and conformational transitions occur at rates which are similar, in agreement with results obtained on membrane-bound enzyme [10]. This result is obtained provided the experimental conditions are identical for the de-occlusion and fluoresecence assays, since there is a marked effect of detergent on the rate of transition at detergent/protein ratios exceeding those required for solubilization. This suggests that high detergent concentrations alter the properties of solubilized enzyme, possibly by changing subunit or lipid–protein interactions. The effects of oligomycin can be interpreted in terms of a simple model, where the de-occlusion of Na^+ is dependent on the de-binding of oligomycin from the enzyme, i.e. oligomycin closes the gate which traps Na^+ in the occluded state.

Methods

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 1160 μmol/mg protein per h at 37°C. Na,K-ATPase activity, phosphorylation capacity and protein content was determined as previously described [13].

Preparation of shark rectal gland enzyme

Na,K-ATPase from the rectal gland from *Squalus acanthias* was prepared as described by Skou and

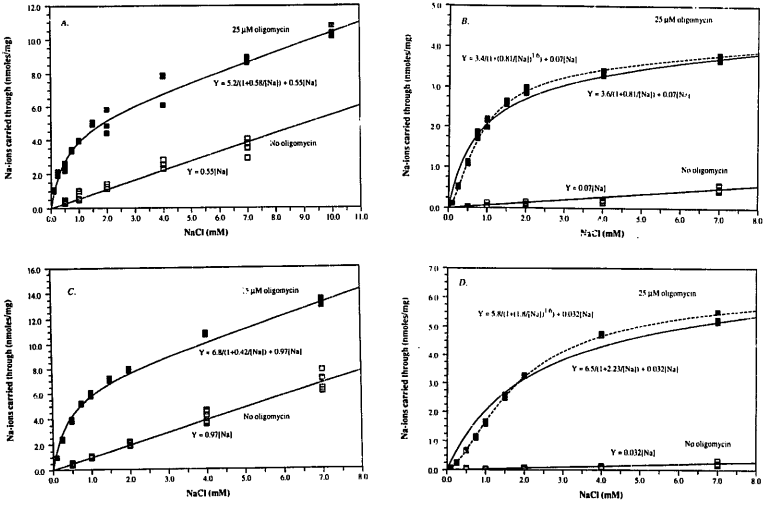

Fig. 1. Occlusion of Na^+ by pig kidney and shark rectal gland Na,K-ATPase. The four panels show the amount of Na^+ (in nmol/mg protein) carried through a cation-exchange resin at 2°C in the absence of oligomycin (open symbols) or in the presence of 25 μM oligomycin (filled symbols) in 30 mM histidine (pH 7.0), 1 mM CDTA and the Na^+-concentrations indicated. Panels A and B show experiments carried out with kidney enzyme (0.9 mg/ml) in the membrane-bound state (panel A) or in the presence of 1.6 mg $C_{12}E_8$/ml (panel B). Panels C and D show the corresponding experiments with 0.9 mg/ml shark rectal gland enzyme in the membrane-bound state (panel C), or at a detergent concentration of 1.7 mg/ml (panel D). Each data point shown represent a single experiment. The curves used to fit the data points for experiments in the absence of oligomycin (open symbols) are straight lines obtained by regression analysis. The curves fitted to the data obtained with oligomycin present are constructed as a sum of the binding in the absence of oligomycin plus a contribution from a binding term of the form $Y = Y_{max}/(1 + (K_{0.5}/[Na^+])^n)$. The values used for Y_{max}, n and $K_{0.5}$ are those deduced from the visually best fit, and are shown in Table I.

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 1760 μmol/mg protein per h. Na,K-ATPase activity, phosphorylation capacity and protein content was determined as previously described [13].

Measurement of Na^+-occlusion using the cation-exchange procedure

The method used is essentially as described by Glynn and Richards [4]. The carboxylic resin Bio-Rad Bio-Rex 70 is equilibrated in 100 mM Tris (pH 7.0), 1 mM CDTA and 0.1 mg/ml $C_{12}E_8$ with 0 or 25 μM oligomycin. Na,K-ATPase (0.9 mg/ml) in 30 mM histidine (pH 7.0) and 1 mM CDTA is incubated with $^{22}Na^+$ at 2 or 6°C in the presence of 25 μM oligomycin (or 0 μM as a control for non-specific binding) and $C_{12}E_8$ as indicated. Solubilization is instantaneous after addition of detergent. About 450 μl is forced through the 1-ml column at 2°C, with the speed of the piston being adjusted to allow the enzyme suspension to be in contact with the resin for times between 0.6 and 2.5 s (this is calculated from the flow-rate and the volume of the liquid-phase in the resin). The amount of ^{22}Na emerging from the column is determined from γ-radiation. The specific radioactivity is adjusted to give about 400 counts per min per nmol Na^+. No determination of protein was done on the effluent from the resin, since it has previously been shown that the loss of protein in the column is negligible [9].

Stopped-flow fluorimetry

Measurements of changes in fluorescence were determined using a SFM-2 stopped-flow apparatus (Biologics, France). Excitation was at 530 nm, and emission was measured with a photomultiplier equipped 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 for the experiments shown in Figs. 5–8 were prepared in the following way: Both stopped-flow syringes contained 30 mM histidine (pH 7.0), 1 mM CDTA and 1 μM 6-carboxyeosin (6-CEo). In addition one syringe contained 0.1 mg Na,K-ATPase protein/ml and 2 mM NaCl. The other syringe contained no protein or Na^+ but 20 mM KCl. Oligomycin was added as an ethanol solution to both syringes. The final ethanol concentration did not exceed 0.5%.

$C_{12}E_8$ was added to give 0.5 mg/ml in both syringes 3–7 min before the stopped-flow experiment was carried out, and was always added before Na^+ and oligomycin.

The volume delivered from each syringe was 150 μl per shot and the flow-time was chosen to be 200 ms. This instrumental setting 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

6-Carboxyeosin was obtained from Molecular Probes, USA. Oligomycin (M_r 790) was obtained from Boehringer-Mannheim, and $C_{12}E_8$ was obtained from Nikko Chemicals, Tokyo. ^{22}Na was obtained from Risø National Laboratories (Denmark).

Results

Quantification of occluded Na^+ in membrane-bound and solubilized Na,K-ATPase

Fig. 1 shows a set of experiments in which the amount of occluded Na^+ is determined for membrane-bound and $C_{12}E_8$-solubilized pig kidney Na,K-ATPase (panels A and B) and shark rectal gland Na,K-ATPase (panels C and D).

The experiments are performed at a low temperature (2°C) in order to decrease the rate of de-occlusion of Na^+. The amount of occluded Na^+ (in nmol/mg) is measured from the radioactivity emerging with the Na,K-ATPase when a sample is forced through a small cation-exchange column (see Methods). The time in which the enzyme is in contact with the column in this experiment is about 1.1 s, which is a short time relative to the half-time for de-occlusion (the de-occlusion rate constant is less than 0.01 s^{-1} for membrane-bound Na,K-ATPase under the present conditions, not shown).

The amount of Na^+ emerging from the column in the absence of oligomycin can be interpreted to be proportional to the Na^+-concentration, suggesting a non-specific binding of Na^+, Fig. 1. Note that the non-specific binding is decreased drastically for both enzymes upon solubilization. This makes the determination of occluded Na^+ more reliable for the solubilized enzymes. As previously observed [7], there is an additional amount of Na^+ carried through the column when the enzyme in Na^+ is equilibrated with oligomycin. This extra Na^+ carried through is termed 'occluded' Na^+, since the rate constant for release is very low. The occlusion clearly shows saturation.

The concentration dependence of the amount of Na^+ occluded for membrane-bound enzyme is fitted by a simple hyperbola ($Y = Y_{max}/(1 + (K_{0.5}/[Na^+])^n)$, with $n = 1.0$). This function is used to describe the data, notably the changes occurring upon solubilization. This equation implies that the enzyme may occlude an number of Na-ions (Y_{max} nmol/mg protein) with a half-

TABLE I

Comparison of kinetic parameters describing Na^+-dependence of oligomycin-induced Na^+-occlusion

The experiments shown in Fig. 1 are fitted by a straight line (non-specific binding in the absence of oligomycin) or by a sum of the non-specific binding and a specific binding term of the form $Y = Y_{max}/(1 + K_{0.5}/[Na^+]^n)$, where Y_{max} is the maximal occlusion, $K_{0.5}$ is the half-maximal-binding constant for Na^+ and n is the Hill coefficient.

Enzyme source	Pig kidney		Shark rectal gland	
State	mem. [a]	solub. [b]	mem. [a]	solub. [b]
Maximal occlusion capacity (Y_{max}, nmol/mg protein)	5.2	3.4	6.8	5.8
Half-maximal-binding constant ($K_{0.5}$, mM)	0.58	0.81	0.42	1.8
Hill coefficient (n)	1.0	1.6	1.0	1.6
Non-specific Na^+-binding (nmol/mg protein per mM Na^+)	0.55	0.07	0.97	0.03
Phosphorylation capacity (nmol/mg protein)	2.0		2.6	

[a] Membrane bound.
[b] Solubilized in $C_{12}E_8$.

maximal binding-constant $K_{0.5}$. The data presented here are fitted satisfactorily by this equation for the membrane-bound enzymes, Figs. 1A and 1C, whereas the fit is not adequate for the solubilized enzymes, Figs. 1B and 1D. Solubilization leads to an observable sigmoidicity of the curves, most predominant with the shark enzyme (Fig. 1D). The sigmoidicity can be taken as a sign of cooperativity, which is reflected in the much better fit by the Hill-equation shown in Fig. 1D with $Y_{max} = 5.8$ nmol/mg, $K_{0.5} = 1.8$ mM and a Hill coefficient $n = 1.6$. A similar analysis for solubilized kidney enzyme (dotted line in Fig. 1B) gives values of $Y_{max} = 3.6$ nmol/mg, $K_{0.5} = 0.81$ mM and $n = 1.6$. Table I summarizes the values for $K_{0.5}$ and Y_{max} for the four experimental conditions. Note that solubilization decreases the maximal amount of Na^+ occluded by the kidney enzyme from 5.2 to 3.4 nmol/mg, with no major change in $K_{0.5}$ (but with an increase in the Hill coefficient to 1.6). For shark rectal gland enzyme the change in occlusion-capacity is less affected by solubilization, but there is a marked shift to a lower affinity for Na^+ ($K_{0.5}$ increases from about 0.4 mM to about 1.8 mM. As with the kidney enzyme, the Hill coefficient increases from 1.0 to 1.6).

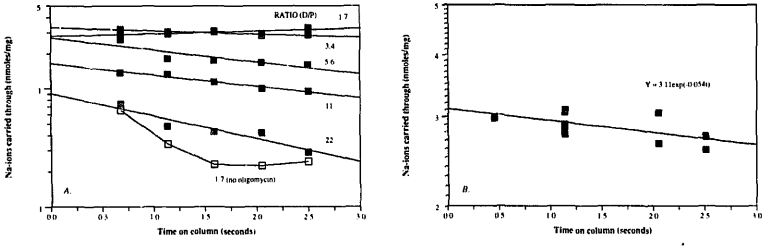

Fig. 2. De-occlusion of Na^+ from solubilized Na,K-ATPase by removal of unbound Na^+. Shown in this figure is the amount of Na^+ (nmol/mg protein, on a log scale) carried through the cation exchange resin when the protein is allowed to be in contact with the resin for times ranging from 0.7 to 2.5 s at 2°C. Panel A shows a set of experiments with pig kidney Na,K-ATPase at a protein concentration of 0.9 mg/ml in 30 mM histidine (pH 7.0), 1 mM CDTA, 2 mM NaCl, $C_{12}E_8$ to give the detergent/protein (D/P) weight ratios shown (D/P between 1.7 and 22) and 25 μM oligomycin (filled symbols) or no oligomycin present (open symbols). Note that the values given with filled symbols are corrected for the amount of Na^+ carried through in the absence of oligomycin. Each point represents the average of two or three experiments. Panel B shows a similar experiment with shark enzyme at a detergent/protein ratio of 1.5, and the data in the logarithmic plot are fitted by a straight line with a slope of 0.054 s^{-1}, intercepting the y-axis at 3.11 nmol/mg. Here each data point represents a single experiment. For the data at 1.1 s the average of five experiments is 2.93 nmol/mg with a S.D. of 0.14 nmol/mg.

Table I also give the steady-state values for maximal phosphorylation, which is taken as a measure of the enzyme concentration. Ratios of 2.6–2.7 mol Na^+ per mol phosphorylation site are obtained, close to the expected three Na^+-sites per phosphorylation site for the membrane-bound enzymes.

Rate of de-occlusion of Na^+ from solubilized Na,K-ATPase determined by removal of unbound Na^+

The rate of de-occlusion of Na^+ can be followed with the cation-exchange technique if the rate constant is in the range 1 to 2 s^{-1} or smaller (this is because our apparatus can be adjusted to let the enzyme be in contact with the cation exchange resin for times in the range 0.6 to 2.5 s). The de-occlusion takes place because free Na^+ is quantitatively bound to the resin during the passage, thus allowing Na^+ to leave the enzyme (de-occlude) with no subsequent occlusion of Na^+ being possible.

For both membrane-bound pig kidney and shark rectal gland enzyme the rate constant for de-occlusion is very small, about 0.01 s^{-1} (experiments not shown). There is thus practically no de-occlusion taking place during the time is takes to remove bulk Na^+ (see below). The time-course of de-occlusion can therefore not be followed with this technique, but the maximal occlusion capacities are conveniently determined. The values of 5.2 and 6.8 nmol Na^+ occluded/mg protein for the membrane-bound enzyme (see Fig. 1 and Table I) can be assumed to reflect the maximal occluding capacity of these enzyme preparations.

For solubilized Na,K-ATPase, the situation is somewhat different. At low concentrations of detergent (detergent/protein ratios of 1 to 2) the rate of de-occlusion for kidney Na,K-ATPase is also very slow, Fig. 2A. However, at higher detergent concentrations (ratios up to 22) rate constants of 0.1 to 0.3 s^{-1} are obtained, indicating that de-occlusion is more rapid at high detergent/protein ratios. Solubilized shark enzyme also has a higher de-occlusion rate constant than the membrane-bound form, Fig. 2B. The rate constant describing the exponential decay shown in Fig. 2B is about 0.05 s^{-1}, but is not very well-determined when the longest time of contact between Na^+ and the cation-exchange resin is 2.5 s.

The maximal occlusion capacity for solubilized enzyme (Table I) can be calculated as follows: The observed value of Y_{max} should be corrected by multiplication of a factor of $\exp(k_{de\text{-}occ} \cdot t)$, where $k_{de\text{-}occ}$ is the rate constant for de-occlusion (see below) and t is the time spent on the column. Since the values for k_{deocc} are about 0.01–0.03 s^{-1} (at low detergent/protein ratios) and t is 1.1 s in the present experiment (Figs. 1B and 1D), the correction factor amounts to at the most only 1.1, i.e. the maximal values for occlusion by solubilized Na,K-ATPase in Fig. 1 and Table I are underestimated by 10% at the most.

Properties of Na^+-occlusion by solubilized kidney Na,K-ATPase

Fig. 3A gives the relationship between occlusion of Na^+, solubilization and the detergent/protein ratio. Analysis of the solubilized protein shows that it is the Na,K-ATPase protein, which is solubiized under these conditions. The values for occluded Na^+ are obtained after subtraction of the amount of Na^+ emerging from the column in the absence of oligomycin. There is a small decrease in the net amount of Na^+ emerging

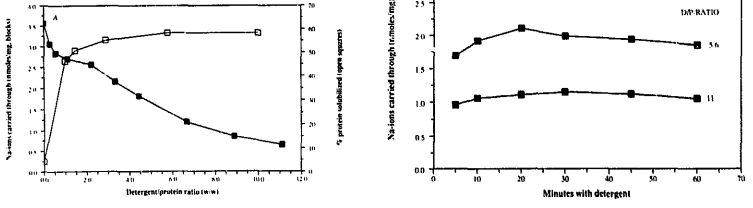

Fig. 3. Detergent-effects on occlusion of Na^+ by kidney Na,K-ATPase. Kidney enzyme was solubilized with $C_{12}E_8$ at the detergent/protein weight ratios indicated, and after addition of Na^+ and oligomycin the amount of Na^+ carried through the column was measured (the filled symbols show net occlusion, i.e. the difference between the amount of Na^+ carried through in the presence and the absence of oligomycin). The protein concentration is 0.9 mg/ml in 30 mM histidine (pH 7.0), 1 mM CDTA, 2 mM NaCl and 25 μM oligomycin. The temperature was 2°C, and the time spent in contact with the cation exchange resin was 1.1 s. Panel A shows the effect in increasing the detergent/protein ratio from 0 (i.e. membrane bound enzyme) to about 11. For comparison is also shown the percentage of total protein solubilized under the same conditions (open symbols). Panel B shows the time-dependence of the amount of occluded Na^+. Note that the data have not been corrected by subtraction of oligomycin-independent Na^+ carried through the column.

from the cation exchange column when low amounts of detergent are added, concomitant with solubilization of the protein. At detergent/protein ratios of 1 to 2, where the Na,K-ATPase is fully solubilized, there is no additional significant reduction in the occlusion capacity (about 2.7 nmol Na^+/mg protein at 2 mM NaCl). The decrease in occlusion at higher detergent-concentrations (detergent/protein ratios exceeding 2, see Fig. 3) can either be due to an inactivation of the occlusion-capability at the point of solubilization or to an increased rate of de-occlusion (since the experiment is done with a fixed time of de-occlusion, 1.1 s, an increase in the rate constant for de-occlusion will lead to lower values of Na^+ occluded). It is thus conceivable that the decrease in occlusion capacity observed upon solubilization (at high detergent/protein ratios) is partly due to the increase in the rate constant for de-occlusion at high detergent/protein ratios (Fig. 2A). The correction factor due to de-occlusion at a detergent/protein ratio of 11 can be calculated (Fig. 2A) to be 1.3 – this brings the value for occlusion up to 1.1 nmol/mg from 0.8, which still is far below optimal occlusion capacity. A third explanation (which has not been investigated) could be that the affinity for Na^+ is decreased as the detergent/protein ratio is increased.

Fig. 3B shows that a solubilized kidney preparation, with solubilization done at a detergent/protein ratio of 5.6 or 11 is stable with respect to ability to occlude Na^+ for at least 60 min at 2°C.

The decrease in occlusion capacity thus probably takes place at the point of solubilization, with the remaining occlusion capacity unchanged with time (on a time-scale of hours at 2°C).

De-occlusion of Na^+ measured after addition of K^+

The low rate of de-occlusion at low detergent/protein ratios makes it possible to follow the effect of addition of K^+ for a given period of time on the amount of Na^+ occluded. If it is assumed that K^+ will bind to unliganded enzyme (forming E_2K, and thus prevent occlusion of Na^+) the rate of de-occlusion of Na^+ can be followed.

Fig. 4 shows a set of such experiments with solubilized shark rectal gland Na,K-ATPase. Clearly K^+ can bring about de-occlusion, probably simply by hindering the occlusion of Na^+ as suggested above. The rate of de-occlusion increases with an increase in the detergent concentration as above (Fig. 2A). An increase from a detergent/protein ratio of 1.5 to 3 increases the rate constant by a factor of two, from about 0.06 to 0.12 s^{-1} at 6°C. The rate of de-occlusion is also temperature-dependent, with an increase in rate constant from 0.035 s^{-1} at 2°C to 0.06 s^{-1} at 6°C, Fig. 4.

The data in Fig. 4 have not been corrected for non-specific binding of Na^+. This is why the equations used to fit the data have a small time-independent

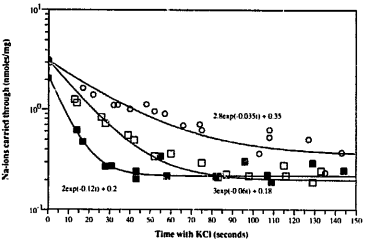

Fig. 4. De-occlusion of Na^+ studied by addition of 20 mM K^+. This figure shows the effect of addition of 20 mM K^+ to $C_{12}E_8$-solubilized shark Na,K-ATPase. Occlusion is induced by oligomycin with incubation at 2 °C for 10–15 min in a medium with a protein concentration of 0.9 mg/ml, 30 mM histidine (pH 7.0), 1 mM CDTA, 2 mM NaCl and 25 μM oligomycin. The temperature of incubation with KCl is 2°C (circles) or 6°C (blocks) and the detergent/protein ratio is 1.5 (open symbols) or 3 (filled blocks). KCl is added as a concentrated solution at time zero, and after the indicated times the suspension is forced through the cation exchange resin, and the amount of Na^+ carried through is determined. The values at time zero are obtained before addition of K^+, and the average of four determinations was to 3.23 nmol/mg (±0.13, open circles), 3.04 nmol/mg (±0.16, open blocks) and 2.07 nmol/mg (±0.29, filled blocks). The suspension is in contact with the resin for 1.1 s, and the time on the column is thus very short compared to the time required K^+ to induce de-occlusion (rate constants 0.03 to 0.12 s^{-1}).

term. The time-dependent part of the equation is taken to be a single-exponential with the rate constants quoted above. Inspection of the data clearly reveals that other models involving a stepwise dissociation of the ions could fit the data equally well (or better). Here a single exponential fit is chosen in order to have a good parameter for comparison with the transient fluorescent data reported below.

Experiments with kidney enzyme similar to those shown in Fig. 4 for shark enzyme gave fluctuating results: most of the Na^+ was de-occluded before the enzyme emerged from the column, but there was a large scatter in the data obtained. There is at present no explanation for the difference between the action of K^+ on the shark enzyme and kidney enzyme.

De-occlusion of Na^+ determined from 6-CEo fluorescence

A fluorescence method for measuring conformational transitions in solubilized Na,K-ATPase has recently been published [14]. This method can be used to monitor the de-occlusion reaction when K^+ is added to Na,K-ATPase with Na^+ occluded. The method relies on 6-CEo having a high fluorescence when bound to the E_1Na form, and a low fluorescence when not bound, i.e. when the enzyme is in the E_2-forms.

The method is more versatile than the cation-exchange technique, since time domains from milliseconds to minutes can be employed, temperature controlled over a wide range, and much less enzyme is required for a measurement of a full time-course. Mixing can also be controlled carefully, allowing a wide variation in ligand-combinations.

Fig. 5 shows the decrease in fluorescence when pig kidney enzyme and shark rectal gland enzyme in the E_1-form with occluded Na^+ (at 2 mM NaCl and with or without 25 μM oligomycin) is mixed with 20 mM K^+ at 6°C. The decrease in fluorescence corresponds to a transfer of the enzyme from the E_1-form to the E_2-form.

In the presence of 25 μM oligomycin the rate constant for the fluorescence decrease is very low for both species, Table II, with shark rectal gland enzyme being more readily transferred ($k_{obs} = 0.054$) to the E_2 state than the pig kidney enzyme ($k_{obs} = 0.014$). These slow rates of transition has previously been demonstrated at room temperature [7], and correspond to the slow de-occlusion of Na^+ from the enzyme when oligomycin is present. In the absence of oligomycin the transition away from the E_1-form is very rapid with rate constants larger than 30 s^{-1} (not shown, see also Refs. 15 and 16).

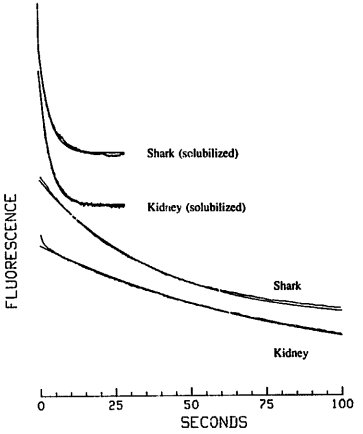

Fig. 5. The effect of solubilization on the rate of de-occlusion of Na^+, as deduced from the rate of change in 6-CEo fluorescence. Na,K-ATPase in 2 mM NaCl and 25 μM oligomycin was mixed with an equal volume of buffer containing 20 mM KCl. The lower fluorescence tracings show experiments with membrane-bound enzymes, and the upper tracings show experiments with $C_{12}E_8$-solubilized enzyme. The buffer contained 30 mM histidine (pH 7.0 at 6°C), 25 μM oligomycin and final concentrations were 0.05 mg protein/ml, 1 μM 6-CEo, 10 mM KCl and 1 mM NaCl and 0 (lower tracings) or 0.5 mg $C_{12}E_8$/ml (upper tracings). The temperature was 6°C. The transients were fitted by a single exponential of the form $F(t) = F(\infty) + F \cdot e^{(-k_{obs} \cdot t)}$, with the values for k_{obs} shown in Table II. Note that the tracings have been normalized to about the same amplitude and displaced vertically to ease comparison.

TABLE II

Rate constants used to fit the transient fluorescent changes shown in Fig. 5 assuming a single exponential of the form $F(t) = F(\infty) + F \cdot e^{(-k_{obs} \cdot t)}$

The experiments were carried out in 30 mM histidine (pH 7.0 at 6°C) with 25 μM oligomycin present and final concentrations were 0.05 mg protein/ml, 1 μM 6-CEo, 10 mM KCl, 1 mM NaCl and 0 (membrane-bound enzyme) or 0.5 mg $C_{12}E_8$/ml (solubilized enzyme). The temperature was 6°C.

Source of Na,K-ATPase	k_{obs} (s^{-1})	
	membrane bound	solubilized
Pig kidney	0.014	0.30
Shark rectal gland	0.054	0.29

Comparison of the results shown in Fig. 5 (Table II) and the measured rates of de-occlusion, Fig. 4, shows that the measured rate of transition away from the E_1-form, supposedly the de-occlusion reaction, is much faster (rate constant 0.3 s^{-1}) than the actual measured rates of de-occlusion, about 0.035–0.1 s^{-1}. As seen below, this discrepancy has two simple explanations, namely that the fluorescence experiments are carried out at a higher detergent/protein ratio (about 10) than the occlusion experiment shown in Fig. 4, and that the temperature for the stopped-flow experiment is 6°C as compared to the 2°C routinely used for the de-occlusion experiments. The following experiments are carried out in order to elucidate the influence of the detergent/protein ratio and temperature on the de-occlusion of Na^+.

The effect of the detergent/protein ratio on the rate of de-occlusion as determined by fluorescence changes

The influence of the detergent/protein ratio on the rate of fluorescence change is determined by addition of increasing amounts of detergent to the enzyme. Measurement of the rate constant for the fluorescence change with detergent concentrations in the range 0–1 mg/ml (giving detergent/protein ratios up to 20),

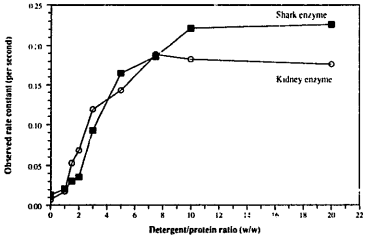

Fig. 6. The effect of detergent on the rate of de-occlusion deduced from stopped-flow fluorescence experiments. Na,K-ATPase in 2 mM NaCl was mixed with an equal volume of buffer containing 20 mM KCl at increasing concentrations of detergent, and the rate of fluorescence decrease is measured. The filled blocks show experiments with shark enzyme, and the open circles are values for pig kidney enzyme. The buffer contained 30 mM histidine (pH 7.0 at 6°C), and final concentrations were 0.05 mg protein/ml, 1 μM 6-CEo, 10 mM KCl, 1 mM NaCl and $C_{12}E_8$ to give the detergent/protein ratio shown. The temperature was 6°C.

shows that there is a marked increase in the rate constant with increasing detergent concentrations at 2°C, Fig. 6. The rate constant is about 0.02 s^{-1} at solubilizing concentrations (about 0.2 mg $C_{12}E_8$/ml), increasing to about 0.2 s^{-1} at high detergent/protein ratios. This is in agreement with the results for the rate of de-occlusion shown in Figs. 2 and 4.

Temperature dependence of the E_1 to E_2 transition at low temperature

The temperature dependence of de-occlusion (an increase in rate constant by a factor of 2–3 from 2°C to 6°C, Fig. 4) can also be seen with the fluorescence technique. Fig. 7 shows the temperature dependence of the rate constant for the fluorescence decrease associated with the E_1Na to E_2 transition for membrane-bound and solubilized Na,K-ATPase. For both the pig kidney (Fig. 7A) and the shark rectal gland enzyme (Fig. 7B), the rate constant increases by a factor of 4–5 when the temperature is increased from 2°C to 12°C. The experiments in the presence of detergent (at detergent/protein ratios of either 4 or 20) show that the temperature dependence is changed slightly by solubilization. The activation energy (estimated from the slopes of the lines in Figs. 7A and 7B) increases from about 20–23 kcal/mol for the membrane-bound enzymes to about 26–30 kcal/mol for the solubilized enzyme. The rate of fluorescence decrease (i.e. the rate of transfer from the E_1 to the E_2-form) is increased drastically by solubilization. Fig. 7 shows an increase by a factor of 20 when the detergent concentration is changed from 0 (membrane-bound enzyme) to 1 mg/ml.

The values for the rate constants for de-occlusion of Na^+ from solubilized shark enzyme (Fig. 4) are also indicated in Fig. 7 (filled diamonds in panel B) to show the agreement between the rate constants derived from fluorescence measurements and the actual de-occlusion rate constants. The rate of fluorescence change is independent of the K^+ concentration in the range

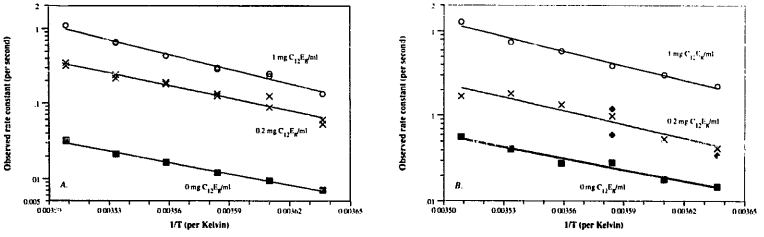

Fig. 7. Temperature dependence of the rate constant for de-occlusion as deduced from fluorescence measurements. This figure shows dependence on reciprocal temperature (K^{-1}) of the rate constant (on a log scale) derived from the fall in fluorescence when Na,K-ATPase in the presence of 2 mM NaCl and 25 μM oligomycin is mixed with 20 mM K^+. The rate constants are derived from single-exponential fits of curves such as the upper tracing in Fig. 8A. The left hand panel (A) shows results with pig kidney enzyme, and the right hand panel (B) shows results with shark rectal gland enzyme. The buffer contained 30 mM histidine (pH 7.0 at 6°C), 25 μM oligomycin, 0.05 mg protein/ml, 1 μM 6-CEo, 10 mM KCl, 1 mM NaCl and 0, 0.2 or 1.0 mg $C_{12}E_8$/ml. Straight lines are fitted through the data, corresponding to activation energies (in kcal/mol) of 22.8 (blocks), 25.8 (crosses) and 30.2 (circles) for panel A and 20.2 (blocks), 24.1 (crosses) and 26.2 kcal/mol (circles) for panel B. The filled diamonds show the values for the rate constant for de-occlusion derived from the experiments in Fig. 4.

from 5 mM to 150 mM (not shown). In the interpretation of the results it does therefore probably not matter that the de-occlusion experiments are carried out in 20 mM K^+ (Fig. 4) and the fluorescence experiments with 10 mM K^+ (Figs. 6 and 7).

Determination of the dissociation constant for oligomycin binding to solubilized Na,K-ATPase

A marked effect of oligomycin on the rate of conversion from E_1Na to E_2K is seen seen in stopped-flow experiments when enzyme with less than optimal oligomycin is mixed with K^+, Fig. 8. Here the fluorescence tracings are clearly two-component at oligomycin-concentrations in the range 0.3 to 1 μM for kidney enzyme and 1–6 μM for shark enzyme, Figs. 8A and 8B, respectively. Intuitively, the fluorescence transients suggest that enzyme with Na^+ bound (but with no oligomycin bound and thus no occlusion of Na^+) is transferred rapidly to the E_2-form by K^+, whereas the reaction of enzyme molecules with oligomycin bound is transferred slowly. The fraction of enzyme with oligomycin bound obviously increases with an increase in the oligomycin concentration, Figs. 8A and 8B.

Figs. 8C and 8D shows the result of an analysis of the transients for the solubilized enzymes: the time-course is fitted with a non-linear least-squares method

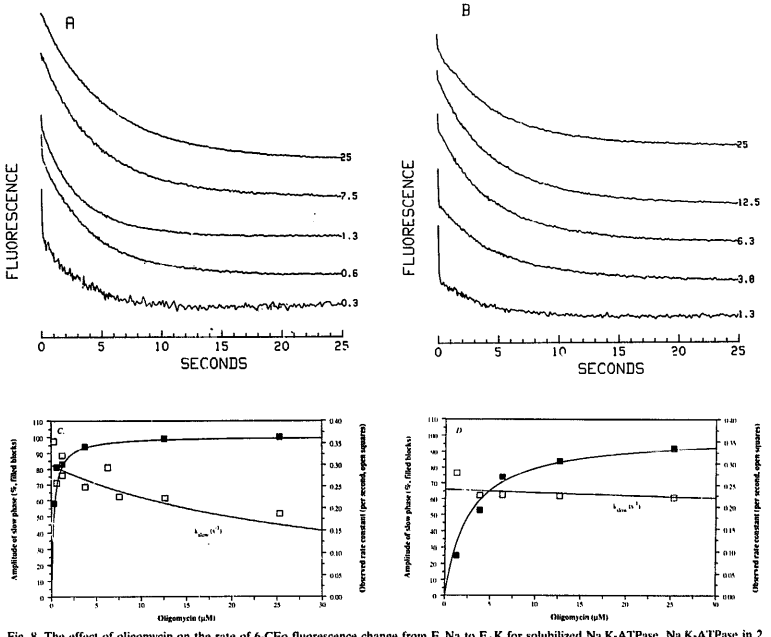

Fig. 8. The effect of oligomycin on the rate of 6-CEo fluorescence change from E_1Na to E_2K for solubilized Na,K-ATPase. Na,K-ATPase in 2 mM NaCl and oligomycin at the indicated concentrations were mixed with an equal volume of buffer containing 20 mM KCl. The left hand panel (A) shows experiments with pig kidney enzyme, and the right hand panel (B) shows experiments with shark rectal gland enzyme. The buffer contained 30 mM histidine (pH 7.0 at 6°C), the indicated concentrations of oligomycin (μM) and final concentrations were 0.05 mg protein/ml, 1 μM 6-CEo, 10 mM KCl, 1 mM NaCl and 0.5 mg $C_{12}E_8$/ml. The temperature was 6°C. Note that the tracings have been normalized to about the same amplitude and displaced vertically to ease comparison. Panels C (kidney enzyme) and D (shark enzyme) shows the dependence of the amplitude of the slow phase (filled blocks) and the observed rate coefficient for the slow phase (open symbols) on the oligomycin concentration when the transients in panels A and B, respectively, are fitted by exponentials of the form $F(t) = F(\infty) + F_{slow} \cdot e^{(-k_{slow} \cdot t)} + F_{rapid} \cdot e^{(-k_{rapid} \cdot t)}$. The lines fitted through the data are obtained from an analysis of the two-pool model shown in Scheme I.

to a sum of two exponentials, one with a large rate constant (held constant at 30 s^{-1}) and one with a much lower rate constant. The percentage of slow fluorescence decay (F_{slow}) increases (and saturates) with increasing oligomycin concentrations, and the rate constant attributed to the slow phase (k_{slow}) decreases from about 0.3 to about 0.15 s^{-1} for kidney enzyme and is fairly constant for shark enzyme (about 0.2 s^{-1}), Figs. 8C and 8D, respectively.

$$\begin{array}{ll} E_1\cdot Na \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} {}^{o}E_1\cdot Na & \text{High fluorescence-forms} \\ \downarrow k_2 & \\ E_2\cdot K & \text{Low fluorescence-form} \end{array}$$

Scheme I

The data in Fig. 8 are interpreted in terms of a simple model (Scheme I). It is assumed that there is an equilibrium between the occluded (oligomycin-bound) form ${}^{o}E_1\cdot Na$ and the non-occluded (oligomycin-free) form $E_1\cdot Na$. The E_1-forms are denoted high fluorescence forms because they both bind 6-CEo with high affinity and 6-CEo is present in these experiments. 6-CEo is omitted from Scheme I for clarity.

It is known that the rate constant for the conformational change E_1Na–E_2K is large ($k_2 \geq 30$ s^{-1}) in the absence of oligomycin [15]. If we then attribute the rate constant k_{-1} to the de-occlusion (and de-binding of oligomycin), and let the rate constant k_1 be proportional to the oligomycin concentration, the amplitude of the slow phase (F_{slow}) and the observed rate constant for the slow phase (k_{slow}) can be interpreted in terms of the rate constants of the model in Scheme I along the lines of the analysis of Klodos et al. (page 468 in Ref. 17). In that paper, explicit equations are given which relate the parameters obtained from a bi-exponential fit (i.e. k_{slow} and F_{slow}) of a 2-pool model to the values of k_1, k_{-1} and k_2.

In Figs. 8C and 8D are shown the data obtained for k_{slow} and F_{slow} from the bi-exponential fit of the transients in Figs. 8A and 8B, respectively, together with the variation of these parameters with the oligomycin concentration using the values given in Table III for k_1, k_{-1} and k_2 (full lines in Figs. 8C and 8D).

TABLE III

Rate constants for binding and dissociation of oligomycin used to fit the data shown in Figs. 8C and 8D to Scheme I

k_1 is the rate constant for binding of oligomycin and has the dimension $M^{-1}s^{-1}$, and k_{-1} is the corresponding dissociation rate constant (s^{-1}). k_2 is the large rate constant reflecting the transformation of E_1Na to E_2K, in these experiments set to 30 s^{-1}. K_o is the dissociation constant calculated for the enzyme-oligomycin complex, i.e. $K_o = k_{-1}/k_1$.

	Kidney enzyme	Shark enzyme
Rate constant for binding (k_1, $M^{-1}s^{-1}$)	$1.0\cdot10^6$	$0.09\cdot10^6$
Rate constant for dissociation (k_{-1}, s^{-1})	0.3	0.24
Dissociation constant for oligomycin (K_o, μM)	0.3	2.7
Rate constant for E_1Na to E_2K transition (k_2, s^{-1})	30	30

Discussion

The purpose of the present paper is to demonstrate that detergent solubilized Na,K-ATPase can occlude Na^+ in the presence of oligomycin, and to investigate the interaction between oligomycin and the solubilized enzyme. The results obtained also suggest that the rate of fluorescence changes associated with conformational changes correlate well with the rate of de-occlusion of Na^+.

Occlusion capacity, cooperativity and de-occlusion rates

The present data for the Na^+-dependence of oligomycin induced occlusion (Fig. 1) compares well with previous work on membrane-bound kidney enzyme, where $K_{0.5}$-values of 0.6–0.9 mM are found [6,18]. Here is found about 0.6 mM for kidney enzyme and 0.4 mM for shark enzyme. The stoichiometry of Na^+-occluded is in the expected range, approaching 3 Na^+-ions per phosphorylation-site (Table I).

The maximal occlusion capacity is decreased only slightly upon solubilization of shark enzyme, from about 2.6 Na^+ to about 2.2 Na^+ per phosphorylation site. It is thus possible to solubilize the Na,K-ATPase and retain almost full occlusion capacity for shark enzyme. For kidney enzyme there is a larger decrease in the occlusion capacity, which can be ascribed to a partial denaturation of the enzyme upon solubilization. The capacity for occlusion is decreased from about 2.6 Na^+ to about 1.8 Na^+ per phosphorylation site. Vilsen et al. [6] has observed a similar decrease in occlusion capacity after solubilization, but they also showed that performing occlusion before solubilization lead to higher values for the amount of Na^+ occluded, which was attributed to instability of the non-occluded form of the enzyme [6]. Here the occlusion experiments are performed at a low temperature, immediately after solubilization (i.e. within 5 min). For shark enzyme, there is no effect of the order of addition of oligomycin and detergent, but for kidney enzyme we observe – in agreement with Vilsen et al. [6] – an increase in the occlusion capacity (25%) by addition of oligomycin before solubilization (experiments not shown). Denaturation of Na,K-ATPase activity has previously been described for solubilized kidney enzyme [19], where a typical bi-phasic response to detergent was seen: about 40% of the Na,K-ATPase was lost rapidly (presumably

at the solubilization step) and the remaining 60% of activity was less prone to detergent inactivation. In the same study shark enzyme was far less susceptible to inactivation, in agreement with the results showed in this paper for the occlusion-capacity of the solubilized enzymes.

Solubilization increases the $K_{0.5}$-value for Na^+ for shark enzyme by a factor of 4–5, whereas $K_{0.5}$ is only increased slightly upon solubilization of kidney enzyme, from about 0.6 to about 0.8. The shape of the curves shown in Fig. 1 indicate that there is a more marked interaction between the occluded ions in the solubilized state, as reflected by the increase in sigmoidicity (and in the Hill coefficient, from 1.0 to 1.6). These changes in mode of interaction has also been observed for example with enzymes modified by trypsinization [8] or by radiation inactivation [22]. In the latter case the cation sites exhibit a marked positive cooperativity after alteration of the protein structure by irradiation. These observations taken together with the present results suggest that the properties of the solubilized enzyme are different from those the native membrane-bound enzymes due to a modification in protein structure, presumably a change of interaction between subunits in the case of the solubilized enzyme.

The rate constant for de-occlusion for membrane-bound enzyme is very low at 2°C (less than 0.01 s^{-1} for kidney enzyme), which has also been observed by Shani-Sekler et al. (a half-time about 9 min, Ref. 18). Solubilization of the enzyme leads to higher rates of de-occlusion, notably at high detergent-protein ratios. At a detergent/protein ratio of 3 we find a rate constant of about 0.02 s^{-1} (Fig. 2A) at 2°C. This is slower than the rate of 0.2 s^{-1} found for de-occlusion of Na^+ in the Cr-ATP complex at 20°C, but the two rates are compatible if an activation energy of about 28 kcal/mol is assumed, which is in the same range as what we find with the fluorescence experiments (Fig. 7).

Temperature dependence of the transition rates

The calculated values for the activation energies of the rate constants for the fluorescence decrease (Fig. 7) for membrane-bound enzyme (20–23 kcal/mol) are about the same as those seen for the de-occlusion of K^+ from FITC-modified Na,K-ATPase [20], and also correspond to the activation energy seen for the Na,K-ATPase reaction for shark enzyme [21].

Interaction of oligomycin with the solubilized enzyme

The model described in Scheme I is a kinetic 'minimal model' used to describe the results of the fluorescence experiments. The implication of the model as it stands is that the slow process of de-occlusion is due to a slow dissociation of oligomycin, which also has been suggested for membrane-bound Na,K-ATPase [23]. Models involving more molecular detail can also fit the data, notably if the step involving binding/dissociation of oligomycin and the step with slow de-occlusion of Na^+ are to be separated (see Scheme II). Here k_1 is taken to be proportional to the oligomycin concentration (dimension $M^{-1}s^{-1}$), but the values of the individual rate constants can not be assigned from the present experiments.

$$\begin{array}{ccccc} E_1\cdot Na & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & {}^*E_1\cdot Na & \underset{k_{de\text{-}occ}}{\overset{k_{occ}}{\rightleftharpoons}} & {}^*E_1\cdot(Na) \quad \text{High fluorescence-forms} \\ \downarrow k_2 & & & & \\ E_2\cdot K & & & & \text{Low fluorescence-form} \end{array}$$

Scheme II

Scheme II is more attractive than Scheme I, especially since the occlusion and slow de-occlusion of Na^+ is seen also with modifiers of the enzyme other than oligomycin [5,6]. The temperature dependence of the observed fluorescence transition, which indicates activation energies in the range 25–30 kcal/mol, also suggest that the reactions involved are more complex than binding and dissociation of oligomycin: the values obtained here are associated with enzyme-catalyzed reactions (see for example Ref. 21) rather than simple ligand-binding and dissociation reactions, for which much lower activation energies are to be expected. The values for the binding and dissociation rate constants derived (k_1 and k_{-1}, Table III) are also much larger than the previously obtained values for dissociation and binding of oligomycin to membrane bound Na,K-ATPase [23]. The large increase in the rate of fluorescence change observed at increasing detergent/protein ratios (Fig. 6) should according to Scheme I imply that the rate of dissociation of oligomycin increases with the detergent/protein ratio, whereas Scheme II allows the effect of detergent to be on the de-occlusion step (i.e. to increase $k_{de\text{-}occ}$).

Correlation between de-occlusion and fluorescence transition rates

It has previously been demonstrated that there is a good correlation between de-occlusion of Rb^+ and fluorescence changes from E_2 to E_1 in dog kidney enzyme [10]. Here it is shown that under appropriate conditions the same correlation is found for detergent solubilized shark enzyme (Fig. 8B). It is, however, evident that there a strong interplay between the detergent and the protein. Notably, a high detergent/protein ratio gives rise to an increase in the de-occlusion rates. If the effect of detergent is taken mainly to transform the enzyme into the monomeric state at high detergent/protein ratios, then a tentative conclusion is that monomeric Na,K-ATPase is different from its oligomeric counterpart in having a larger rate constant for de-occlusion of Na^+ as well as a larger degree of

positive cooperativity between the ions occluded. Another possibility is that a high detergent concentration perturbs the lipid–protein interaction, leading to changes in protein dynamics (i.e. increased rates of conformational changes, Fig. 7) and subsequent to denaturation of the enzyme (Fig. 3A), as has been suggested for Ca^{2+}-ATPase [24].

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