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CONFORMATIONAL TRANSITIONS OF DETERGENT-SOLUBILIZED Na,K-ATPase ARE CONVENIENTLY MONITORED BY THE FLUORESCENT PROBE 6-CARBOXY-EOSIN

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SUMMARY : 6-carboxy-eosin is introduced as a sensitive, non-covalently bound fluorescent probe for monitoring conformational changes in detergent-solubilized Na,K-ATPase. The dissociation constant for 6-carboxy-eosin is about 0.1 μM in 20 mM NaCl at 6 °C (pH 7.0) for Na,K-ATPase solubilized in $C_{12}E_8$. It is shown that the slow conformational change from E_2 (in K^+) to E_1 (in Na^+) is 4-fold more rapid in the solubilized state than in the membrane-bound state, both for shark rectal gland and pig kidney Na,K-ATPase. The rate of the E_1 to E_2 transition is rapid and of the same order of magnitude both for the membrane-bound and the solubilized enzyme. All conformational transitions are considerably slower for pig kidney enzyme than for shark enzyme, both in the membrane-bound and in the solubilized state.

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).

Structural and kinetic studies of the properties of the Na,K-ATPase have been carried out with a variety of experimental approaches and techniques, including studies of active, detergent-solubilized Na,K-ATPase (2-4). The advantage of using solubilized enzyme is that the relationship between enzyme activity and the oligomeric structure of the protein/detergent complex can be determined using conventional techniques such as analytical ultracentrifugation (2,3) and size-exclusion HPLC (5).

Studies of Na,K-ATPase kinetics invariably rely on determination of conformational states of the enzyme under a variety of conditions, and determination of rates of transition between these states (6). The major protein conformations in the non-phosphorylated state are E_1 (predominant in NaCl) and E_2 (predominant in KCl). Na,K-ATPase in the E_1-form binds ATP, ADP and the fluorescent dye eosin with high affinity, whereas the enzyme has low affinity for the nucleotides and eosin in the E_2-form. The advantage of using eosin as a probe for enzyme conformations is that the fluorescence yield of eosin is increased several-fold upon binding to the enzyme (7), i.e. transitions between the two enzyme conformations can be easily followed by fluorimetry.

ABBREVIATIONS

$C_{12}E_8$: octaethyleneglycoldodecylmonoether; E_1 : the Na,K-ATPase conformation predominant in NaCl; E_2 : the Na,K-ATPase conformation predominant in KCl.

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The purpose of the present paper is primarily to introduce a very sensitive fluorescent probe, 6-carboxy-eosin, which can be used to distinguish conformational states in detergent-solubilized Na,K-ATPase. Such a probe has not previously been available.

6-carboxy-eosin behaves essentially as eosin when used with membrane-bound enzyme, the affinity for 6-carboxy-eosin being higher than for eosin (not shown). In detergent solution, however, the fluorescence yield of free (non-bound) eosin is increased to a level comparable to that of protein-bound eosin, i.e. the ability to discriminate between bound and free eosin using a fluorimeter is lost. Eosin can thus not be used to monitor conformational changes in detergent-solubilized Na,K-ATPase. In contrast to this, the fluorescence yield of free 6-carboxy-eosin in detergent solution is about the same as in the absence of detergent, i.e. the ability to discriminate between free and protein-bound fluorescent probe is retained in the presence of detergent. In the present paper stop-flow measurements of 6-carboxy-eosin fluorescence is used to monitor the kinetics of the conformational transitions in solubilized Na,K-ATPase, using $C_{12}E_8$ as a detergent in which the enzyme can be solubilized in an active form. It is shown that the major conformations are preserved upon solubilization of Na,K-ATPase from both a mammalian species, pig kidney, and from an elasmobranch, the dogfish rectal gland, and that the rate of transition from the E_2-form to the E_1-from is increased 4-fold upon solubilization.

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 (8) 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 hour at 37 °C.

Preparation of Shark Rectal Gland Enzyme. Na,K-ATPase from the rectal gland of Squalus acanthias was prepared as described by Skou and Esmann (9), 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 hour. Na,K-ATPase activity and protein content was determined as previously described (10).

Stop-flow fluorimetry. Measurements of changes in fluorescence were done 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. Signal-to-noise was reduced by digitally adding 3-5 tracings. Non 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) at 6 °C. In addition one syringe contained 0.1 mg protein/ml and either 20 mM NaCl (experiments shown in Figure 2 and 4) or 2 mM KCl (experiments shown in Figure 3). The other syringe contained in addition to buffer either 20 mM NaCl (Figure 2), 40 mM NaCl (Figure 3) or 20 mM KCl (Figure 4). 6-carboxy-eosin was added as indicated in the legends to the figures. $C_{12}E_8$ was added to give 0.5 mg/ml in both syringes 3-7 minutes before the stop-flow experiment was carried out in the experiments involving solubilized enzymes. At detergent/protein ratios (w/w) above two all the Na,K-ATPase protein is solubilized, and solubilization is instantaneous (2).

The volume delivered from each syringe was 150 μl per shot, the flow-time being 100 ms. This gives a dead time of less than 2 ms in this apparatus, which is sufficiently short for the reactions to be followed in these experiments.

Materials. Eosin was obtained from Koch-Light. 5-(6-)-carboxy-eosin is a mixture of the two isomers, and can be obtained from Molecular Probes, USA. The isolated 5- and 6-carboxy-

eosins were custom-synthesized by Molecular Probes, USA. $C_{12}E_8$ was obtained from Nikko Chemicals, Tokyo.

RESULTS AND DISCUSSION

The chemical structures of eosin and the two analogues, 5- and 6-carboxy-eosin, are shown in Figure 1. Note that the carboxyl groups are fully deprotonated at pH 7.0, at which the experiments in the present paper are carried out. The advantage of using carboxy-eosin instead of eosin probably stems from a decreased ability of carboxy-eosin to partition into the detergent micelles : the fluorescence of eosin is increased dramatically by addition of detergent as soon as the critical micelle concentration is reached, whereas the fluorescence of both 5- and 6-carboxy-eosin is virtually unaffected up to 1 mg $C_{12}E_8$/ml (not shown).

In the following experiments only 6-carboxy-eosin is used, since 6-carboxy-eosin gives fluorescence responses which are about a factor 3 larger than 5-carboxy-eosin due to a higher affinity for the eosin-binding site (not shown).

Determination of the affinity of 6-carboxy-eosin for solubilized Na,K-ATPase.

Figure 2 shows a set of experiments used to determine the affinity of 6-carboxy-eosin for $C_{12}E_8$-solubilized shark Na,K-ATPase in the presence of 20 mM NaCl at pH 7.0 at 6 °C. If 6-carboxy-eosin (here denoted CEo) interacts with the Na,K-ATPase (E) according to a simple binding/dissociation reaction:

$$E + CEo \underset{k_{-1}}{\overset{k_1}{<\!\!-\!\!-\!\!-\!\!>}} E{\bullet}CEo \qquad (1)$$

an exponential increase in fluorescence should be obtained when enzyme is mixed with 6-carboxy-eosin. This is what is observed for 6-carboxy-eosin in the range 0.25 to 1 μM, see the upper 4 tracings in Figure 2. The observed rate constant (k_{obs}) would be related to the 6-carboxy-eosin concentration in the following way (see refs. 11-13):

$$k_{obs} = k_{-1} + k_1{\bullet}[CEo]$$

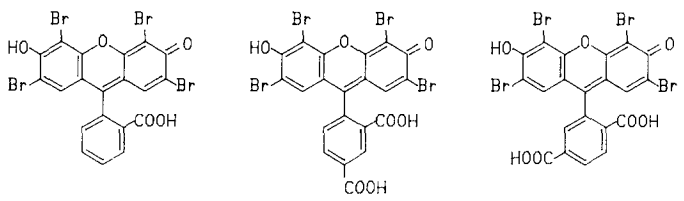

Figure 1. Chemical structures of eosin, 5-carboxy-eosin and 6-carboxy-eosin in their fully protonated states.

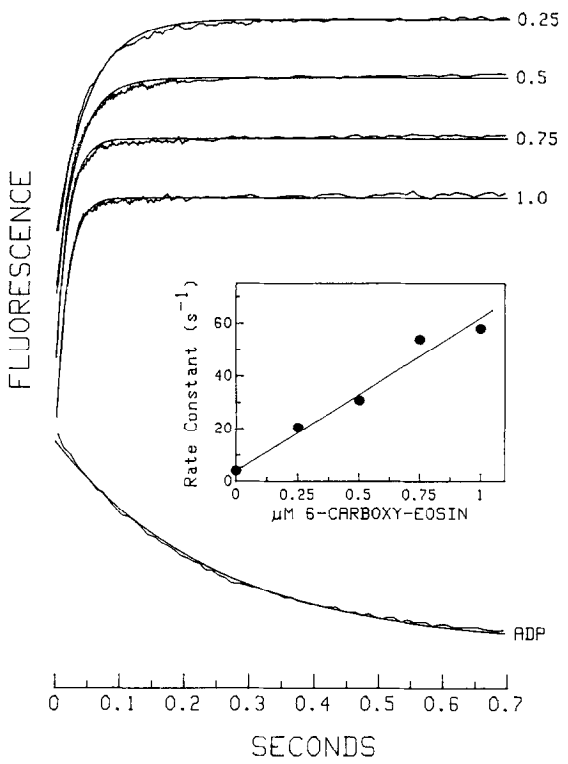

Figure 2. Binding and dissociation of 6-carboxy-eosin from $C_{12}E_8$-solubilized shark rectal gland Na,K-ATPase. For the experiments shown in the upper four tracings Na,K-ATPase in 20 mM NaCl was mixed with an equal volume of buffer containing 20 mM NaCl and 6-carboxy-eosin to give the indicated final concentrations (in μM). The lower tracing represents the decrease in fluorescence when Na,K-ATPase in the presence of 20 mM Na^+ and 1 μM 6-carboxy-eosin is mixed with 200 μM ADP in 20 mM NaCl. The buffer contained in all 5 experiments 30 mM histidine (pH 7.0 at 6 °C) and 0.5 mg $C_{12}E_8$/ml, and the final protein concentration was 0.05 mg/ml. The temperature was 6 °C. The transients were fitted by exponentials of the form $F(t) = F(\infty) - F_1 \cdot e^{(-k_{obs} t)}$ (in the present analysis the small, slow rise in fluorescence is ignored - it can be attributed to a slow non-specific binding of dye (not shown)). The insert shows the relationship between the observed rate constant (k_{obs}) and the 6-carboxy-eosin concentration (see text). Note that the transients have been normalized to the same amplitude, and are displaced vertically to ease comparison.

where k_{-1} has the dimension s^{-1} and k_1 the dimension $M^{-1} \cdot s^{-1}$. This relation is shown in the insert in Figure 2, where the regression line corresponds to a value of $63 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ for k_1 and 4.0 s^{-1} for k_{-1}. The dissociation constant for 6-carboxy-eosin is then $k_{-1}/k_1 \sim 0.06$ μM.

A decrease in fluorescence - due to displacement of 6-carboxy-eosin - is observed when saturating ADP is added to enzyme with 6-carboxy-eosin bound (lower tracing in Figure 2), and the rate of fluorescence decrease determined from the tracing is 3.9 s^{-1}, in good agreement with the value for k_{-1} (intercept of the regression line at the y-axis in the insert). The higher affinity for 6-carboxy-eosin than for eosin probably stems from the decreased rate-constant for dissociation (cf. ref. 13).

Ligand-induced conformational changes monitored by 6-carboxy-eosin.

Figure 3 shows a set of experiments in which Na,K-ATPase suspended in 2 mM KCl is exposed to a final concentration of 20 mM NaCl (and 1 mM KCl). The fluorescence of 6-carboxy-eosin increases, reflecting transfer of Na,K-ATPase from the E_2-form to the E_1-form, which binds 6-carboxy-eosin with high affinity. The transition rate is at least a factor 10 higher for shark enzyme (Figure 3A) than for kidney enzyme (Figure 3B), and for both enzymes the rates are increased upon solubilization in 0.5 mg $C_{12}E_8$/ml, cf. Figure 3 and Table 1. The transition from E_2 to E_1 can be adequately fitted by two exponentials for shark enzyme, whereas the kidney enzyme transition can be fitted by a single exponential. The increased rate of transition upon solubilization is reflected as an increase in the rate-constant. For shark enzyme the rate constant associated with the major component is increased 4 fold (note that bi-exponential fitting of data which gives rate constants differing only by a factor of 3-5 is by no means un-equivocal). For kidney enzyme it is simple, since the data are fitted by a single exponential, the rate of which is increased by a factor of 4 upon solubilization.

The reverse reaction, from E_1 to E_2, can also be monitored by 6-carboxy-eosin. Figure 4 shows the fluorescence tracings when Na,K-ATPase in 20 mM NaCl is mixed with KCl to give a final concentration of 10 mM KCl (and 10 mM NaCl). As expected (12-14), the rates

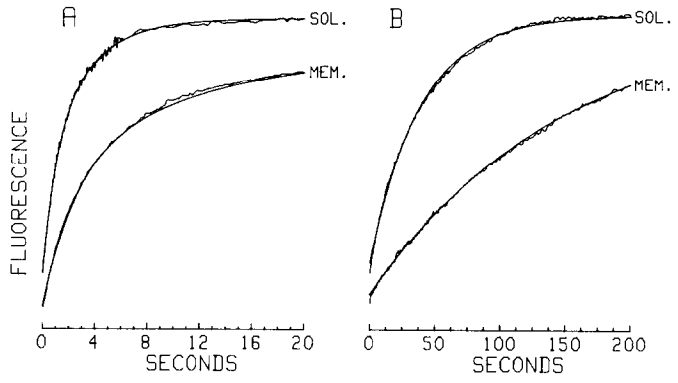

Figure 3. Transition from the E_2 to the E_1-form monitored by the 6-carboxy-eosin fluorescence. Na,K-ATPase in 2 mM KCl was mixed with an equal volume of 40 mM NaCl. The lower tracing shows the fluorescence change of membrane-bound enzyme (MEM.), and the upper tracing is obtained for detergent-solubilized enzyme (SOL.). The left hand panel (A) shows experiments with shark enzyme, and the right hand panel (B) shows experiments with kidney enzyme. The buffer was 30 mM histidine (pH 7.0 at 6 °C), 1 μM 6-carboxy-eosin, and final concentrations were 0.05 mg protein/ml, 1 mM KCl and 20 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 exponentials of the form $F(t) = F(\infty) + F_1 \cdot e^{(-k1 \cdot t)} + F_2 \cdot e^{(-k2 \cdot t)}$, using the coefficients given in Table 1. Note that the transients have been normalized to the same amplitude, and are displaced vertically to ease comparison.

TABLE 1

This table contains the rate constants used to fit the transient fluorescent changes shown in Figures 3 and 4. For transients fitted by two exponentials ($F(t) = F(\infty) + F_1 \cdot e^{(-k1 \cdot t)} + F_2 \cdot e^{(-k2 \cdot t)}$), the relative proportions F_1 and F_2 (in %) are also given. Membranous enzyme is abbreviated Mem. and $C_{12}E_8$-solubilized enzyme Sol.

	Species	State	k_1 (s^{-1})	F_1	k_2 (s^{-1})	F_2
E_2 to E_1 (Figure 3)	Shark	Mem.	0.85	54%	0.084	46%
		Sol.	1.00	45%	0.31	56%
	Kidney	Mem.	0.0067	100%		
		Sol.	0.026	100%		
E_1 to E_2 (Figure 4)	Shark	Mem.	~100	53%	9.6	47%
		Sol.	85	63%	18	37%
	Kidney	Mem.	1.7	75%	0.92	25%
		Sol.	2.4	76%	0.68	20%

are much more rapid than for the E_2 to E_1 transition, and shark enzyme has again a considerably higher rate of conformational change, cf. Table 1. The transition from E_1 to E_2 can be adequately fitted by two exponentials for both membrane-bound and solubilized enzymes. Solubilization with $C_{12}E_8$ does not alter the rate of fluorescence change appreciably, there is only a minor increase in the rate-constant attributed to the slow component (see Figure 4 and Table 1).

It can be concluded that solubilization in $C_{12}E_8$ leads to an 4-fold increase in the E_2 to E_1 transition, but not to a comparable increase in the E_1 to E_2-transition. The solubilized

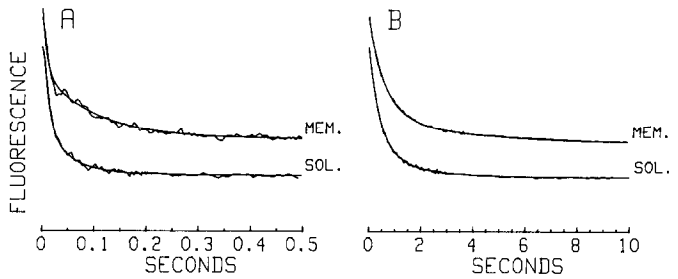

Figure 4. **The effect of detergent on the rate of transition from E_1 to E_2. Na,K-ATPase in 20 mM NaCl was mixed with an equal volume of buffer containing 20 mM KCl in the absence of detergent (upper tracing) or in the presence of 0.5 mg $C_{12}E_8$/ml (lower tracing). The left hand panel (A) shows experiments with shark enzyme, and the right hand panel (B) shows experiments with kidney enzyme. The buffer contained 30 mM histidine (pH 7.0 at 6 °C), 1 μM 6-carboxy-eosin and final concentrations were 0.05 mg protein/ml, 10 mM KCl and 10 mM NaCl and 0 (upper tracings) or 0.5 mg $C_{12}E_8$/ml (lower tracings). The temperature was 6 °C. The transients were fitted by exponentials of the form $F(t) = F(\infty) + F_1 \cdot e^{(-k1 \cdot t)} + F_2 \cdot e^{(-k2 \cdot t)}$, using the coefficients given in Table 1. Note that the transients have been normalized to the same amplitude, and are displaced vertically to ease comparison.**

Na,K-ATPase is thus poised more towards the E_1-form in the solubilized state than in the membrane-bound state, in agreement with previously obtained kinetic data on cation activation of enzymatic activities of $C_{12}E_8$-solubilized Na,K-ATPase (cf. Table 1 in ref. 17).

CONCLUSIONS

6-carboxy-eosin is introduced as a sensitive tool for probing conformational states and transitions of detergent-solubilized Na,K-ATPase. The main advantage of using this fluorescent probe is that the enzyme is retained in its native state (i.e. does not require covalent modification as with for example fluorescein isothiocyanate (14, 15) or 5-acetamidofluorescein (16)), and the probe can be used both with membrane-bound and solubilized pig kidney or shark rectal gland enzyme. It should be noted that eosin (and thus also 6-carboxy-eosin) has the disadvantage of acting as a nucleotide-analog with respect to binding, and effects of ADP or ATP on conformational transitions can thus not be followed with the eosin-technique.

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