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FLUORESCENT LABELING OF $(Na^+ + K^+)$-ATPase BY 5-IODOACETAMIDOFLUORESCEIN

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5-Iodoacetamidofluorescein (5-IAF) covalently labels dog kidney $(Na^+ + K^+)$-ATPase with approximately 2 moles incorporated per mole of enzyme. ATPase and K^+-phosphatase activities are fully retained after reaction, and the kinetic parameters for Na^+, K^+, Mg^{2+}, ATP and *p*-nitrophenyl phosphate are likewise not significantly affected. The fluorescence of the bound 5-IAF is increased by ATP, Na^+, and Mg^{2+}, and decreased by K^+. These fluorescence changes likely reflect ligand-induced stabilization of the E_1 or E_2 states of the enzyme.

The $(Na^+ + K^+)$-ATPase functions in cell membranes to couple sodium and potassium transport to ATP hydrolysis, thereby maintaining ionic gradients. Current formulations of the transport mechanism predict cyclic interconversions between at least two different conformational states of the enzyme: $E_1 \cdot Na$ and E_2 (K) [1,2]. Transitions between these states (and their substates) have recently been studied using a number of techniques. One of the most sensitive is fluorescence, either intrinsic protein fluorescence [3] or extrinsic probes introduced as substrate analogs or modifying reagents (see, for example Refs. 4–7). The usefulness of these studies has been limited either by small fluorescence changes or, in the case of probes, inhibition of enzyme activity. Castro and Farley [8] found that reaction of iodoacetate with $(Na^+ + K^+)$-ATPase does not affect enzymatic activity; furthermore it is localized on the catalytic subunit close to the phosphorylation site. Therefore we looked for a fluorescent alkyl halide with similar specificity that could function as a reporter group without inhibiting the enzyme. In this report, we describe the reaction of $(Na^+ + K^+)$-ATPase with the fluorescent probe, 5-iodoacetamidofluorescein (5-IAF) with high sulfhydryl specificity [9]. 5-IAF labeled enzyme is fully active, and has a strong fluorescence signal that responds to ligands that shift the enzyme between E_1 and E_2 states.

$(Na^+ + K^+)$-ATPase (spec. act. 15–26 μmol P_i/min per mg protein) was purified from dog kidney outer medulla with SDS using the procedure of Jørgensen [10]. Protein was measured by the method of Lowry et al. [11] using bovine serum albumin as a standard. $(Na^+ + K^+)$-dependent ATPase and K^+-phosphatase activities were measured as previously described [12]. Reaction with 5-IAF was performed using 1.5 mg enzyme with 100 μM 5-IAF in 50 mM imidazole-HCl (pH 7.5) containing 20 mM KCl in a total volume of 0.5 ml, in the dark, for 24–48 h at 4°C. The reaction was stopped by passage over Sephadex G-25 in 50 mM imidazole-HCl (pH 7.5). Controls run in the absence of 5-IAF did not lose activity by this treatment. The amount of 5-IAF bound was de-

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Abbreviations: $(Na^+ + K^+)$-ATPase, sodium plus potassium dependent adenosine triphosphatase (EC 3.6.1.3); K^+-phosphatase, K^+-dependent phosphatase; 5-IAF, 5-iodoacetamidofluorescein; SDS, sodium dodecyl sulfate; FITC, fluorescein isothiocyanate; $K_{0.5}$, concentration of substrate, activator or inhibitor required to produce half-maximal effect.

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termined spectrophotometrically in 50 mM imidazole, 0.1 mM EDTA (pH 7.5) with 1% SDS using an extinction coefficient determined for the free probe in the same buffer at 492 nm of 76 990 $cm^{-1} \cdot M^{-1}$. Fluorescence was measured in imidazole-EDTA with 10–20 μg/ml enzyme in a total volume of 2.2 ml using an Aminco-Bowman spectrofluorimeter equipped as previously described [12]. The fluorescence titrations were performed at 4°C in order to (1) minimize hydrolysis of ATP and (2) increase the fluorescence intensity. Excitation was at 490 nm and emission at 520 nm. SDS gel electrophoresis was performed using precast 7.5% gels and BioPhore SDS buffer purchased from BioRad. Gels were either stained with Coomassie blue, or photographed under ultraviolet illumination using Kodak Pan 2415 film and an orange filter.

With the mild conditions used, approx. 2 mol of 5-IAF are incorporated irreversibly per mol of $(Na^+ + K^+)$-ATPase (Table I). ATP does not reduce binding significantly, while incubation of 5-IAF with dithiothreitol or mercaptoethanol before reaction with the enzyme reduces incorporation almost to zero. It appears then that the 5-IAF binds to groups on the enzyme that are not protected by ATP, i.e., they are somewhat removed from the active site. Since blocking the reactive group of the probe with small thiols abolishes labeling, it is likely that 5-IAF reacts with groups on the protein component of the ATPase (presumably sulfhydryl residues) rather than non-specifically absorbing into the lipid matrix.

When examined on SDS gel electrophoresis, the labeled enzyme has only one fluorescent band, at the position of the catalytic (α) subunit, which runs at an M_r of 96 000 in our system (Fig. 1). If, as has been proposed, the functional enzyme contains two α subunits [13], it is tempting to speculate that the labeling stoichiometry is one per subunit; however, we have not yet characterized the binding sites.

Modification with 5-IAF does not inhibit either $(Na^+ + K^+)$-ATPase or K^+-phosphatase activity to any significant extent (Table II). As we intended to make use of the 5-IAF labeled enzyme to study binding of substrates and ions, we decided to determine whether the modification had any effect on the affinities for these in the enzymatic activity. Kinetic parameters were determined under standard assay conditions except for varying the ion or substrate in question. The data were fitted by computer to reciprocal plots or Hill plots, and the $K_{0.5}$ or $K_{m,app}$ calculated according to the best fit. Virtually identical results are obtained for control and modified enzyme for Na^+ and K^+ activation of ATPase activity and for K^+ activation and Na^+ inhibition of K^+-phosphatase activity (Table II).

Hill coefficients for Na^+ and K^+, determined from the same data, are likewise unchanged by the modification ($n = 1.6–1.8$ in all cases). The only values affected to any degree by the labeling are

TABLE I

5-IAF LABELING OF $(Na^+ + K^+)$-ATPase

Reaction conditions	Moles 5-IAF bound per mole enzyme [a]
Control	–
0.1 mM 5-IAF	2.16
0.1 mM 5-IAF + 7 mM ATP	1.95
0.1 mM 5-IAF + 0.2 mM dithiothreitol	0.06
0.1 mM 5-IAF + 0.2 mM mercaptoethanol	0.11

[a] Calculated assuming a molecular weight for the enzyme of 280 000. Results are average values of at least two experiments.

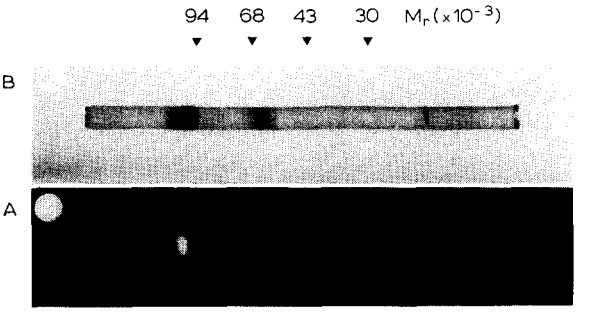

Fig. 1. SDS-polyacrylamide gels of 5-IAF labeled $(Na^+ + K^+)$-ATPase. (A) Unstained gel, photographed as described in the text, (B) Coomassie blue stained gel. 12 μg of enzyme were dissolved in 1 mM Tris-EDTA containing 1% SDS and 40 mM dithiothreitol and applied in duplicate to gels. Electrophoresis was for 4 h at 7–8 mA per gel. The positions of molecular weight markers run in parallel are shown at the top.

TABLE II

ENZYMATIC PROPERTIES OF CONTROL AND 5-IAF LABELED ($Na^+ + K^+$)-ATPase

The units for $V_{max,app}$ are μmol P_i or *p*-nitrophenol/min per mg; for $K_{0.5}$ and $K_{m,app}$, mM. Values presented are averages of duplicate determinations. pNPP, *p*-nitrophenyl phosphate.

Enzyme preparation	Kinetic parameter							
	($Na^+ + K^+$)-ATPase				K^+-Phosphatase			
	$V_{max,app}$	$K_{0.5}$ (Na^+)	$K_{0.5}$ (K^+)	$K_{m,app}$ (ATP)	$V_{max,app}$	$K_{0.5}$ (K^+)	$K_{0.5}$ [a] (Na^+)	$K_{m,app}$ (pNPP)
Control	21.2	10.3	1.0	0.30	4.22	1.6	37.9	2.2
5-IAF labeled	20.2	10.7	0.9	0.51	4.15	1.1	41.4	3.4

[a] Inhibition of K^+-phosphatase by Na^+.

those for ATP and *p*-nitrophenyl phosphate: the $K_{m,app}$ for these are both increased slightly, by less than 2-fold, raising the possibility that the 5-IAF is having some influence on the affinities for these substrates. The effect, though, is certainly a minor one.

The fluorescence properties of the modified enzyme suggest that this probe, like FITC [5] and intrinsic protein fluorescence [3], is monitoring transitions between E_1 and E_2 states of the enzyme induced by specific ligands. Addition of Na^+, ATP, or Mg^{2+} to enzyme in imidazole buffer increases the fluoresence (maximal increases, as determined by titration, are 28% for Na^+, 8% for ATP and 14% for Mg^{2+}) consistent with these ligands favoring the E_1 state. K^+, on the other hand, when added to enzyme in the absence of other ions decreases the signal (by 9% maximally) consistent with it producing the E_2 conformation. At saturating Na^+ concentrations no additional fluorescence increase can be induced by ATP, and ATP or Na^+ antagonizes K^+ quenching of fluorescence (data not shown). In addition, K^+ added after Na^+ reverses the Na^+-induced increase in fluorescence. Therefore it appears likely that the 5-IAF is reporting conformational shifts induced by ATP and ions binding to specific sites on the enzyme.

The observation that the maximal fluorescence changes with sodium and ATP are different suggests that we may be detecting subordinate states of the E_1 conformation as also observed previously [14,15].

In addition, when fluorescence is measured in tris buffer instead of imidazole, at the same pH, the initial fluorescence intensity is higher; the percent increases with sodium and ATP are less and the affinity for K^+, as determined by fluorescence titration, is lowered. These results are consistent with previous reports that Tris has a sodium-like effect on the enzyme [16,17].

The 5-IAF modified enzyme is particularly useful since it is fully active and, interaction with ATP, Mg^{2+}, Na^+ and K^+, not being perturbed, can be readily studied using the relatively large fluorescence changes they induce. For example, addition of ATP to modified enzyme increases the fluorescence in a saturable manner (Fig. 2A). Without any added ligands, a K_d for ATP of 0.12 ± 0.10 μM ($n = 6$) is obtained; addition of 20 mM K^+ increases the K_d for ATP 260-fold to 31.2 ± 12.6 μM ($n = 7$) (Fig. 2B). The titration with ATP is homogeneous, with no evidence of cooperativity. Ouabain + Mg^{2+} + P_i, as well as vanadate + Mg^{2+} abolish the fluorescence changes. These results are in excellent agreement with previously published values for nucleotide binding obtained using other methods [4,7,18–20]. We are currently examining binding of other ligands using this technique.

In summary then, 5-IAF, a fluorescent iodoacetamide derivative, binds covalently to ($Na^+ + K^+$)-ATPase on the catalytic subunit at sites which do not inhibit the ($Na^+ + K^+$)-ATPase or *p*-nitrophenylphosphatase activities or change to any significant extent the kinetic parameters for

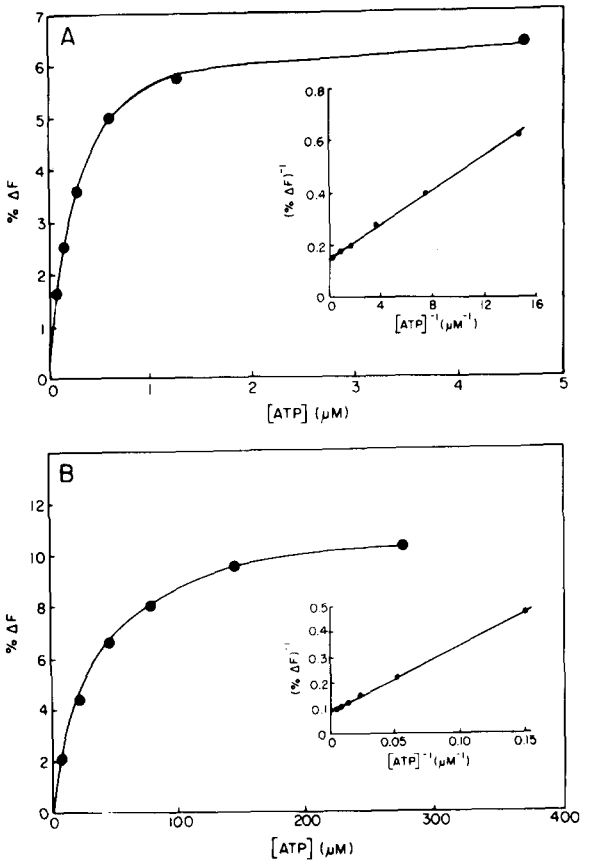

Fig. 2. Fluorescence titration of 5-IAF labeled $(Na^+ + K^+)$-ATPase. (A) In the absence, and (B) in the presence of 20 mM KCl. Insets: Double reciprocal plots of the data. Conditions are as described in the text.

these reactions. The fluorescence changes are large enough that the binding of a number of interesting ligands can be studied with relative ease using a standard fluorimeter. Although more experiments are needed to elucidate the sites of binding of 5-IAF to the $(Na^+ + K^+)$-ATPase, it is likely that this preparation will be useful in studying ligand binding and structural transitions under conditions of turnover.

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