Articles

Spatial Relationship and Conformational Changes between the Cardiac Glycoside Site and β-Subunit Oligosaccharides in Sodium plus Potassium Activated Adenosinetriphosphatase†

Jonathan A. Lee‡ and P. A. George Fortes*
Department of Biology, University of California, San Diego, La Jolla, California 92093
Received July 2, 1986; Revised Manuscript Received August 29, 1986

ABSTRACT: (Na,K)-ATPase, the enzyme responsible for active transport of Na and K across the plasma membranes of animal cells, consists of a catalytic subunit (α) and a glycoprotein subunit (β) with unknown function. We have determined the distance between fluorescent probes directed to specific sites on the α- and β-subunits and ligand-induced changes in the fluorescence of a probe specifically attached to the β-subunit. The cardiac glycoside site on the α-subunit was labeled with anthroylouabain [Fortes, P. A. G. (1977) *Biochemistry 16*, 531–540]. The oligosaccharides on the β-subunit were labeled with lucifer yellow carbohydrazide [Lee, J. A., & Fortes, P. A. G. (1985) *Biochemistry 24*, 322–330]. Resonance energy transfer from anthroylouabain to lucifer yellow was measured by steady-state and time-resolved fluorescence spectroscopy. The distance between these probes was determined from the efficiency of energy transfer. The average distance between anthroylouabain and lucifer yellow was 47 Å and was independent of the number of acceptor molecules attached to the β-subunit. The measured distance corresponds to the distance between the cardiac glycoside site and the center of the labeled oligosaccharides on the β-subunit within one αβ dimer. The distance was the same (47 Å) when anthroylouabain was bound with ATP or P_i as phosphorylating ligands but increased to 49 Å in the presence of vanadate. The change in average distance provides quantitative evidence of a conformational difference between the complexes of cardiac glycosides with (Na,K)-ATPase induced by phosphorylating ligands or by vanadate. Conformational changes induced by ions, substrates, or inhibitors of the enzyme were not detectable by changes in the spectroscopic parameters of lucifer yellow attached to galactose residues of the β-subunit. Ligands that cause two-dimensional crystallization of (Na,K)-ATPase quenched the fluorescence of lucifer yellow, suggesting that crystallization involves extensive interactions between β-subunits.

(Na,K)-ATPase[1] generates and maintains electrochemical gradients of Na^+ and K^+ across the plasma membranes of animal cells by coupling ion pumping to the hydrolysis of ATP. The enzyme consists of two types of subunits (Kyte, 1971), the α-subunit with M_r 112000 (Shull et al., 1985; Kawakami et al., 1985) and the β-subunit, a glycoprotein with a protein mass of 35000 daltons (Noguchi et al., 1986). The stoichiometry of the α- and β-subunits is 1:1 (Craig & Kyte, 1980). The heterodimer of α- and β-subunits is sufficient to account for full catalytic activity and ion pumping (Moczydlowski & Fortes, 1981b; Brotherus et al., 1981; Craig, 1982; Karlish & Kempner, 1984; Fortes & Han, 1985). The α-subunit contains the sites for cardiac glycosides (Ruoho & Kyte, 1974) and phosphorylation (Kyte, 1971) on the extracellular and intracellular sides, respectively, and undergoes ligand-dependent conformational changes thought to be associated with ion transport [see Glynn (1985) and Jørgensen (1986) for recent reviews]. For these reasons, the α-subunit is considered the catalytic subunit. The β-subunit is in close proximity to the cardiac glycoside site because it can be labeled with photoaffinity derivatives of digitoxin bound to the α-subunit (Hall & Ruoho, 1980). The function of the β-subunit is unknown. The observations that ligands of the enzyme alter the susceptibility of the β-subunit to proteolytic enzymes (Lo & Titus, 1978; Koepsell, 1979) suggest that it may undergo conformational changes associated with (Na,K)-ATPase function.

Information on the structure of (Na,K)-ATPase and on the location and magnitude of the conformational changes associated with ion pumping can be obtained from measurements of resonance energy transfer between fluorescent probes attached to specific sites of the enzyme. Toward this goal, work in this laboratory has focused on the development of site-directed fluorescent probes of (Na,K)-ATPase. Fluorescent probes directed to the ouabain (Fortes, 1977; Moczydlowski & Fortes, 1981c) and ATP sites (Moczydlowski & Fortes, 1981a; Karlish, 1980; Fortes & Han, 1985), to sulfhydryl groups on the α-subunit (Jesaitis & Fortes, 1980; Moczydlowski & Fortes, 1981c), and to the membrane lipids (Moczydlowski & Fortes, 1981c; Chong et al., 1985) have been used in energy-transfer measurements. These studies have provided

† This work was supported by NIH Grants HL20262 and RR08135.
‡ Present address: Department of Biochemistry, Roche Institute of Molecular Biology, Nutley, NJ 07110.

[1] Abbreviations: (Na,K)-ATPase, sodium plus potassium activated adenosinetriphosphatase; ANS, 1-anilinonaphthalene-8-sulfonic acid; AO, anthroylouabain; CDTA, *trans*-1,2-diaminocyclohexanetetraacetic acid; EDTA, ethylenediaminetetraacetic acid; lucifer, lucifer yellow carbohydrazide, 4-amino-*N*-[(hydrazinocarbonyl)amino]-2,3-naphthalimide-3,6-disulfonate; Mops, 3-(*N*-morpholino)propanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Tes, 2-[[tris(hydroxymethyl)methyl]amino]-ethanesulfonic acid.

0006-2960/86/0425-8133$01.50/0

information on the distances between the ouabain site and intracellular sites (Jesaitis & Fortes, 1980; Fortes et al., 1981; Carilli et al., 1982) and on the distances of closest approach between sites on the protein and hydrophobic membrane probes (Moczydlowski & Fortes, 1981c) and between ouabain sites on different α-subunits (Moczydlowski & Fortes, 1981c; Chong et al., 1985).

Here we present experiments designed to study the spatial relationships between the α- and β-subunits of (Na,K)-ATPase and possible conformational changes in the β-subunit that might provide clues to elucidate its role in (Na,K)-ATPase function. We measured resonance energy transfer between anthroylouabain (AO), a specific probe of the cardiac glycoside site (Fortes, 1977), and the oligosaccharides of the β-subunit labeled with lucifer yellow (Lee & Fortes, 1985a). We compared the efficiency of energy transfer between the above probes, under different ligand conditions that induce a high affinity for cardiac glycosides, to see if conformational differences between complexes of AO with the enzyme could be detected and quantified. In addition, we tested the effects of the various substrates and inhibitors of the enzyme on the fluorescence of lucifer-labeled oligosaccharides in an attempt to detect possible conformational changes in the β-subunit induced by ligands of the enzyme. A preliminary communication of parts of this work has been presented (Lee & Fortes, 1983).

Materials and Methods

Enzyme Preparation and Assay. (Na,K)-ATPase was purified from dog kidney, and the β-subunit was labeled with lucifer after treatment with galactose oxidase and neuraminidase as described previously (Lee & Fortes, 1985a). The lucifer labeling stoichiometry was altered by varying the extent of oxidation and the temperature and duration of the reaction with lucifer. (Lee & Fortes, 1985a). The (Na,K)-ATPase specific activity ranged from 25 to 32 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$ as determined by the oxidation of NADH in a coupled enzyme assay at 37 °C (Moczydlowski & Fortes, 1981b). There were no significant differences between the specific activities of control and lucifer-labeled preparations.

AO Binding. The concentrations of active sites were determined by fluorescence titrations with AO in 4 mM $MgCl_2$, 3 mM Tris–P_i, 1 mM EDTA, and 50 mM Pipes–Tris, pH 7.0 at 37 °C (Lee & Fortes, 1985a; Fortes, 1986). In the energy-transfer experiments, AO binding was promoted by incubation with Mg + Na_3VO_4, Mg + P_i or Na + MgATP, as described in the legend to Table II. The rate constants for AO association (k_{on}) and dissociation (k_{off}) under the above ligand conditions were determined as described previously (Moczydlowski & Fortes, 1980; Fortes & Lee, 1984; Lee & Fortes, 1985a). The equilibrium dissociation constant (K_D) for AO under each ligand condition was estimated from the ratio k_{off}/k_{on}. The concentration of bound AO, $[AO]_B$, was calculated (Fortes, 1986) from the concentration of added AO, $[AO]_T$, and binding sites, $[E]_T$, with

$$[AO]_B = [AO]_T + [E]_T + K_D - [([AO]_T + [E]_T + K_D)^2 - 4[AO]_T[E]_T]^{1/2}/2 \quad (1)$$

The concentrations of active sites and AO were adjusted so that usually 92–99% of the added AO was bound. In the presence of MgATP + Na, 86% of the added AO was bound because of the larger K_D with these ligands [see Results and Lee and Fortes (1985a)]. Nevertheless, even under the least favorable binding conditions more than 98% of the AO fluorescence was from bound AO because of the large increase in the quantum yield of AO upon binding (Fortes, 1977, 1986).

Fluorescence Measurements. Corrected fluorescence spectra, titrations, binding kinetics, and steady-state anisotropies were measured in a Perkin-Elmer MPF-4 spectrofluorometer as described previously (Fortes, 1977, 1986; Moczydlowski & Fortes, 1980; Lee & Fortes, 1985a). The quantum yield of bound AO, Φ_{AO}, was determined at 24 °C from corrected emission spectra with excitation at 368 nm through a Corning 7-51 filter. The absorbance of the samples was less than 0.05 to prevent inner filter effects. The standards were AO in ethanol (Φ = 0.37; P. A. G. Fortes, unpublished results) and ANS in ethanol (Φ = 0.37; Stryer, 1965). The quantum yield of bound AO was calculated (Shinitzky, 1972) by

$$\Phi_{AO} = \Phi_S \frac{F_{AO}}{F_S} \frac{A_S}{A_{AO}} \left(1 - \frac{r_{AO}}{4}\right) \quad (2)$$

where Φ_S in the quantum yield of the standard, F_i are the areas under the emission spectra, A_i are the absorbances at the exciting wavelength, and r_{AO} is the anisotropy of bound AO. The same value of Φ_{AO} was obtained with both standards.

Time-resolved fluorescence measurements were done by the single photon counting technique with an Ortec nanosecond fluorometer equipped with a high-pressure hydrogen arc lamp (Yguerabide, 1972). Fluorescence decays of AO bound to unlabeled and to lucifer-labeled (Na,K)-ATPase were determined with excitation through a Corning 7-60 filter and emission through a 463-nm interference filter. Preliminary measurements showed that the decay curves were identical when measured without polarizers or with vertically polarized excitation and the emission polarizer set at 0°, 54.7°, or 90°, inidicating that rotational motions of AO were too fast to contribute to the measured decays. Therefore, the AO decay curves for lifetime measurements were recorded without polarizers to increase the signal intensities. Blank decay curves were recorded in the absence of AO with the unlabeled and lucifer-labeled enzymes, and their amplitudes were scaled to those of the corresponding decay curves in the presence of AO by using the ratio of the steady-state intensities in the presence and absence of AO as the scaling factor. The intensities of the blank curves were 8.3% and 37.3% of the intensities in the presence of AO with the unlabeled and the lucifer-labeled (Na,K)-ATPases, respectively. The AO fluorescence decays $I(t)$ were obtained by subtracting the normalized blank decay curves from the corresponding decays in the presence of AO. The lamp pulse $L(t)$ was measured with a scattering solution in the absence of the emission filter. Because of the finite duration of the lamp pulse, the recorded $I(t)$ is related to the actual decay of intensity $F(t)$ by the convolution integral:

$$I(t) = \int_0^t L(T)F(t - T)\, dT \quad (3)$$

$F(t)$ was obtained by deconvolution of $I(t)$ with the method of moments (Yguerabide, 1972), assuming that $F(t)$ was represented by the sum of two exponentials:

$$F(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} \quad (4)$$

where a_i is usually interpreted to be a measure of the fraction of the population that decays with lifetime τ_i and $a_1 + a_2 = 1$. Convolution of $F(t)$ with $L(t)$ generates a new function, $C(t)$, which can be compared to the observed signal $I(t)$. The values of a_i and τ_i were chosen by iteration to reduce the normalized residual χ^2/N to a minimum:

$$\frac{\chi^2}{N} = \frac{1}{N - n} \sum \frac{1}{\sigma_i^2} [I(t_i) - C(t_i)]^2 \quad (5)$$

where σ_i is the standard deviation of $I(t_i)$ due to noise, N is the total number of data points, and n is the number of fitted parameters. The time shift between $L(t)$ and $I(t)$ due to the spectral response of the photomultiplier tube was corrected by shifting the lamp pulse by 0.27 ns.

Decays of anisotropy of bound AO and of lucifer were measured by recording fluorescence decays with vertically polarized excitation and the emission polarizer oriented to transmit light polarized vertically, $I_{\parallel}(t)$, or horizontally, $I_{\perp}(t)$. The decays of lucifer anisotropy were measured with excitation and emission through Corning 5-58 and 3-69 filters, respectively. Blank curves were subtracted, and the amplitudes of $I_{\parallel}(t)$ and $I_{\perp}(t)$ were scaled by the ratio of their steady-state intensities, as described above. The time-dependent anisotropies, $r(t)$, were calculated from the polarized decay curves, without deconvolution, by

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} \quad (6)$$

Energy-Transfer Measurements. For donor quenching experiments, the efficiency of energy transfer E is given by

$$E = 1 - \frac{\tau_{DA}}{\tau_D} \quad (7)$$

and

$$E = 1 - \frac{F_{DA}}{F_D} \quad (8)$$

where τ_{DA} and τ_D are the fluorescence lifetimes of the donor in the presence and absence of acceptor, respectively; F_{DA} and F_D are the steady-state fluorescence intensities of the donor in the presence and absence of acceptor, respectively. The efficiency of energy transfer by sensitized emission of the acceptor was calculated by

$$E = \frac{F_{DA} - F_A - F_D}{(A_D/A_A)F_A - F_D} \quad (9)$$

where F_{DA}, F_A, and F_D, are the fluorescence intensities of the samples with donor + acceptor, acceptor, and donor, respectively, with excitation at the absorption maximum of the donor and emission at acceptor wavelengths; A_D and A_A are the absorbance of the donor and the acceptor at the excitation wavelength (Fairclough & Cantor, 1978). A_D was determined from the concentration of bound AO with an extinction coefficient of 7800 M^{-1} cm^{-1} at 368 nm (Fortes, 1986), which assumes that the extinction coefficient of bound AO is the same as that in ethanol. A_A was determined from the concentration of lucifer in lucifer-labeled (Na,K)-ATPase with an extinction coefficient of 2430 M^{-1} cm^{-1} at 368 nm.

The average distance $\langle R \rangle$ between a single donor (AO) and the center of an ensemble of acceptor molecules was calculated (Gennis & Cantor, 1972) by

$$\langle R \rangle = R_0 \left(N \frac{1 - E}{E} \right)^{1/6} \quad (10)$$

where N is the number of acceptors per enzyme molecule, E is the efficiency of energy transfer at a given N, and R_0, the distance between donor and acceptor at which the energy-transfer efficiency is 50%, is given by (Förster, 1948, 1960)

$$R_0 = [(8.71 \times 10^{23}) J \kappa^2 n^{-4} \Phi]^{1/6} \quad (11)$$

where n is the refractive index of the medium (1.4 for proteins), Φ is the quantum yield of the donor in the absence of acceptor, and κ^2 is an orientation factor that depends on the relative orientation of the transition moments of the donor, the acceptor, and the vector joining them; J is the overlap integral between the donor emission spectrum $F_D(\lambda)$ and the acceptor absorption spectrum $\epsilon_A(\lambda)$:

$$J = \frac{\int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 \, d\lambda}{\int F_D(\lambda) \, d\lambda} \quad (12)$$

J was summed over 5-nm intervals of the corrected emission spectrum of AO and the absorption spectrum of lucifer.

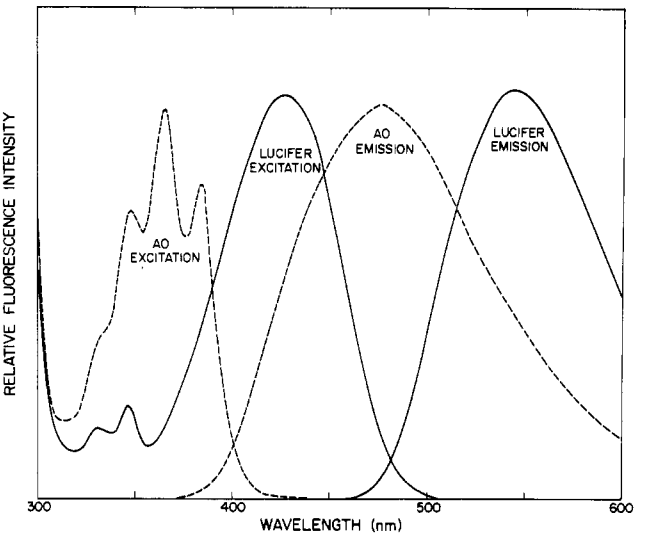

FIGURE 1: Excitation and emission spectra of AO and lucifer in (Na,K)-ATPase. Corrected spectra were recorded at 24 °C in solutions containing 3.5 mM P_i–Tris, 4 mM $MgCl_2$, 1 mM EDTA, 50 mM Mops–Tris, pH 7.0, and either 0.1 μM AO + 0.144 μM unlabeled (Na,K)-ATPase or 30 μg/mL lucifer-labeled (Na,K)-ATPase containing 1.9 μM lucifer. Light scattering and background fluorescence, recorded in the absence of AO, were subtracted from the spectra in the presence of AO. The excitation and emission spectra were normalized to the same peak amplitude.

RESULTS

Distance between Lucifer and Anthroylouabain Bound to (Na,K)-ATPase. Previous studies of AO binding to lucifer-labeled (Na,K)-ATPase showed that the quantum yield of AO decreased in proportion to the number of lucifer molecules attached to the β-subunit, suggestive of resonance energy transfer from AO to lucifer (Lee & Fortes, 1985a). The overlap between the AO emission spectrum and the absorption spectrum of lucifer-labeled (Na,K)-ATPase (Figure 1) indicated that these probes should be an efficient donor–acceptor couple for resonance energy transfer studies. The value of R_0 = 29 Å was obtained with eq 11 by using the measured values of $J = 1.46 \times 10^{-14}$ cm^6 $mmol^{-1}$ and $\Phi_{AO} = 0.27$ and assuming a random orientation between AO and lucifer ($\kappa^2 = 2/3$).

Figure 2 shows emission spectra (with excitation at the absorption maximum of AO) of lucifer-labeled (Na,K)-ATPase with or without bound AO and unlabeled enzyme with bound AO. The fluorescence intensity of AO bound to lucifer-labeled (Na,K)-ATPase decreased compared with that of AO bound to unlabeled enzyme. At wavelengths longer than 480 nm, the fluorescence intensity of the lucifer-labeled (Na,K)-ATPase with bound AO was larger than the sum of the spectra of lucifer and AO after quenching (Figure 2). The decreased fluorescence of AO bound to lucifer-labeled (Na,K)-ATPase and the sensitized emission of lucifer when AO was bound demonstrate resonance energy transfer from AO to lucifer. The transfer efficiencies calculated from AO quenching (37.5 ± 2.1%) or sensitized emission of lucifer (32.8 ± 5%) were not significantly different.

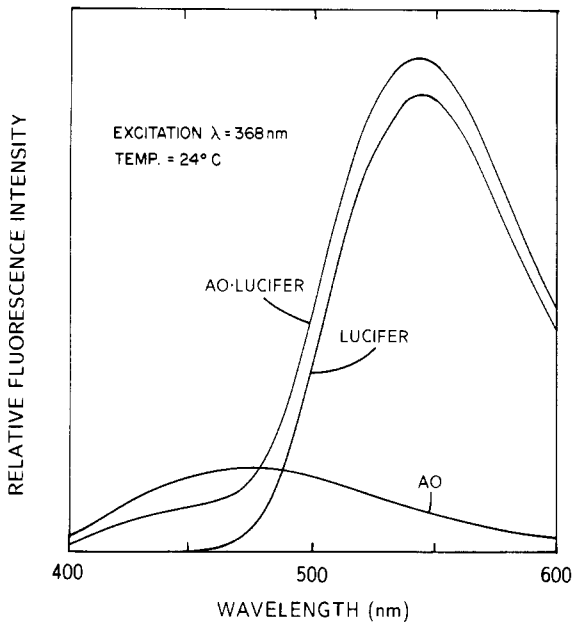

FIGURE 2: Fluorescence energy transfer between AO and lucifer in (Na,K)-ATPase. Corrected emission spectra of 0.144 μM unlabeled (Na,K)-ATPase and 0.154 μM lucifer-labeled (Na,K)-ATPase (62.2 nmol of lucifer/mg of protein) were recorded in the presence and absence of 0.1 μM AO. Blank spectra recorded with unlabeled enzyme without AO were subtracted. Conditions are as in Figure 1.

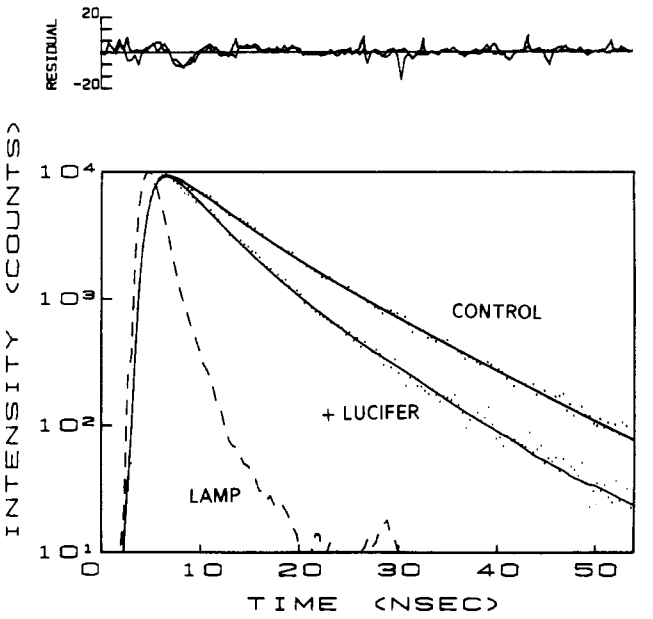

FIGURE 3: Fluorescence decays of AO bound to unlabeled and to lucifer-labeled (Na,K)-ATPase. AO (2.6 μM) was bound to 2.85 μM unlabeled (Na,K)-ATPase (0.71 mg of protein/mL) or 2.85 μM lucifer-labeled (Na,K)-ATPase (0.79 mg of protein/mL, 62.2 nmol of lucifer/mg of protein) in 5 mM P_i–Tris, 5 mM $MgCl_2$, and 50 mM Pipes–Tris, pH 7.0. The temperature was 23 ± 2 °C. The dashed line is the lamp pulse. The points are the experimental curves. The solid lines are the analyzed functions convoluted to the lamp pulse: (control) $F(t) = 0.485 \exp[-(t/3.7)] + 0.515 \exp[-(t/10.1)]$; (lucifer labeled) $F(t) = 0.677 \exp[-(t/3.1)] + 0.323 \exp[-(t/7.2)]$. The χ^2/N values for the fitted functions were 5.1 and 6.6 for the control and lucifer-labeled samples, respectively.

In order to estimate the distance between AO and lucifer, it was necessary to ascertain that lucifer labeling was random, so that all the β-subunits adjacent to AO binding sites were labeled with lucifer. Because of the inherent averaging in steady-state fluorescence methods, experiments such as those shown in Figure 2 cannot be used to know whether the observed energy transfer reflected a homogeneous population or if it resulted from the average of populations of AO molecules highly quenched by acceptors in close proximity and unquenched AO molecules without nearby acceptors. To distinguish between these possibilities, we measured fluorescence decays of AO bound to control and to lucifer-labeled (Na,K)-ATPases.

The fluorescence decays of AO bound to both unlabeled and lucifer-labeled (Na,K)-ATPase were nonexponential (Figure 3). The cause of these nonexponential decays is not clear. The fluorescence decay of AO in solvents can be fit to a single exponential (P. A. G. Fortes, unpublished results), and the kinetics of AO binding and dissociation (Fortes & Lee, 1984; Lee & Fortes, 1985a,b), as well as equilibrium AO binding (Fortes, 1986), are consistent with a homogeneous cardiac glycoside site. The nonexponential decay of AO bound to dog kidney (Na,K)-ATPase could reflect relaxation of the molecular microenvironment of the AO site, rapid interconversion between different environmental states, or sampling of different microenvironments due to motions of AO in the binding site during the excited state. Further work is necessary to interpret the nonexponential decay. For the present purposes, however, a quantitative comparison of the fluorescence decays of AO bound to unlabeled and to lucifer-labeled enzyme gives an independent measure of the energy-transfer efficiency and can provide information on whether all the (Na,K)-ATPase molecules with bound AO were labeled with lucifer.

The fluorescence decays were analyzed assuming they could be described by the sum of two exponentials. The two-exponential fits agreed reasonably well with the experimental curves (Figure 3), as judged by the residual values (5.1 and 6.6 in the absence and presence of lucifer, respectively) and the random deviation across most of the time axis, although a small nonrandom deviation at early times suggests the presence of an additional component. Since the reason for the nonexponential decays is not known and fits to three exponentials increase the uncertainties in the values of the individual components, the two-exponential fits were considered adequate.

Both lifetimes of AO bound to lucifer-labeled (Na,K)-ATPase decreased compared to the control enzyme, and the fraction that decayed with the shorter lifetime increased from 0.48 to 0.68 (Figure 3). The change in the preexponential terms is consistent with the possible causes of the nonexponential decays mentioned above. The decrease in both AO lifetimes excludes the possibility that the steady-state measurements were the average of unquenched and highly quenched AO molecules and indicates that the AO populations with short and long lifetimes participated in energy transfer to lucifer.

To obtain the energy-transfer efficiency from the lifetime measurements, the average lifetimes of AO ($\langle\tau\rangle$) were calculated by

$$\langle\tau\rangle = a_1\tau_1 + \alpha_2\tau_2 \qquad (13)$$

The average lifetimes of AO were 7.0 ± 0.1 and 4.7 ± 0.1 ns in the absence and presence of lucifer, respectively. The energy-transfer efficiency determined from the ratio of the average lifetimes was 33.6 ± 1.5%, which agreed with the values determined by steady-state fluorescence measurements described above.

The anisotropy of bound AO decayed within 0.4 ns (the time resolution of the instrument) to a constant value of 0.14 (data not shown). Therefore, bound AO underwent rapid, restricted motions. Assuming that AO moved over the surface or the volume of a cone, the half-angle of the cone was calculated (Dale & Eisinger, 1975) to be 25° or 33°, respectively, from

Table I: Dependence of AO → Lucifer Energy Transfer on Lucifer Stoichiometry[a]

lucifer stoichiometry[b]			
nmol/mg	mol of lucifer/mol of β	energy-transfer efficiency[c] (%)	average distance[d] (Å)
5.8	1	5.7	46
6.4	1.2	6	47
9.7	1.8	9	47
25	4.5	21	47
62.2	11	37.5	47

[a] AO binding to lucifer-labeled and unlabeled (Na,K)-ATPase was promoted by incubation with 4 mM $MgCl_2$, 3.3 mM Tris-P_i, and 1 mM EDTA in 50 mM Mops-Tris (pH 7.0) at 24 °C. The concentrations of enzyme active sites and AO were adjusted so that more than 98% of the AO fluorescence was from specifically bound AO. [b] Determined from the average of triplicate protein determinations and duplicate lucifer determinations as previously described (Lee & Fortes, 1985a). The moles of lucifer per mole of β was calculated from the nanomoles of lucifer per milligram of protein assuming that the protein was 100% pure and the molecular weight of $\alpha\beta$ = 180 000. [c] Determined by fluorescence quenching of AO as described in the text. Each entry is the average of two to four spectra. [d] Determined by assuming $\kappa^2 = 2/3$.

Table II: Effect of Ligands on AO → Lucifer Energy Transfer[a]

	energy-transfer efficiency		
ligands	donor quenching (%)	sensitized emission (%)	average distance (Å)
MgATP + Na	37.3 ± 2.4	39.8 ± 6.1	47
P_i + Mg	37.5 ± 2.1	32.8 ± 5.0	47
VO_4^{3-} + Mg	32.1 ± 1.9[b]	32.1 ± 7.7	49

[a] Corrected emission spectra, as in Figure 2, were recorded in the presence of 0.1 μM AO and 0.15–0.16 μM unlabeled or lucifer-labeled (Na,K)-ATPase (62.2 nmol of lucifer/mg of protein) at 24 °C. The incubation medium contained 1 mM EDTA-Tris, 50 mM Mops-Tris, pH 7.0, and, where indicated, either 46 mM NaCl, 2.6 mM ATP, and 8 mM $MgCl_2$, 3.3 mM P_i-Tris and 4 mM $MgCl_2$, or 20 μM Na_3VO_4 and 4 mM $MgCl_2$. Two to four spectra were recorded under each ligand condition. The average ± SD energy-transfer efficiencies were calculated as described under Materials and Methods from measurements of the intensities in the presence and absence of lucifer, determined every 5 nm between 420 and 470 nm for donor quenching and every 10 nm between 500 and 600 nm for sensitized emission. The average distance was calculated from the donor quenching data assuming $\kappa^2 = 2/3$. [b] Significantly different ($P < 0.01$ in a nonpaired t test) from the energy-transfer efficiencies in the presence of either P_i or ATP.

the anisotropy decay data and the fundamental anisotropy of AO measured in propylene glycol at −50 °C. The anisotropy of lucifer-labeled (Na,K)-ATPase decayed to a constant value of 0.1 with $t_{1/2}$ = 2–3 ns (data not shown), indicating that significant motions of lucifer occurred within the AO lifetime. Therefore, both donor and acceptor molecules showed considerable motional freedom with rates faster than the energy-transfer rate. These observations justify the assumption of random orientations between AO and lucifer in the calculation of the distance between these probes.

The energy-transfer efficiency and the estimated distance between AO and lucifer as a function of the lucifer stoichiometry are shown in Table I. The transfer efficiency increased with increasing lucifer stoichiometry, reflecting the increased probability of energy transfer as the number of acceptors per donor molecule increased. The calculated distance between AO and lucifer was 47 Å and was independent of the lucifer stoichiometry over an 11-fold range (Table I). These results are consistent with random labeling with lucifer of the β-subunit oligosaccharides. The calculated distance corresponds to the distance between the cardiac glycoside site on the α-subunit and the center of the lucifer ensemble on the β-subunit.

Effect of Ligands on Resonance Energy Transfer from AO to Lucifer. High-affinity binding of cardiac glycosides to (Na,K)-ATPase can be induced by different combinations of ligands, mainly Na + MgATP, Mg + P_i, and Mg + vanadate (Matsui & Schwartz, 1968; Albers et al., 1968; Schwartz et al., 1968; Sen et al., 1969; Post et al., 1969; Fortes, 1977; Wallick et al., 1977; 1979; Hansen, 1979; Moczydlowski & Fortes, 1980). Measurements of the AO association and dissociation rate constants at 24 °C in the presence of these ligand combinations showed that the value of k_{on} was largest with vanadate (2.65×10^4 M^{-1} s^{-1}) and smallest with ATP (6.1×10^3 M^{-1} s^{-1}), whereas k_{off} was largest with ATP (6.5×10^{-5} s^{-1}) and smallest with vanadate (1.97×10^{-5} s^{-1}). The K_D values derived from the kinetic measurements were 0.75 nM with vanadate, 2–5 nM with P_i, and 10.6 nM with ATP. The kinetic differences between these ligands suggested that the conformations of the resulting AO complexes might differ. Therefore, it was of interest to compare the efficiencies of energy transfer from AO to lucifer when AO binding was promoted by different ligands.

Table II lists the AO → lucifer energy-transfer efficiencies determined under different ligand conditions. For each of the ligands tested, the energy-transfer efficiencies determined by donor quenching and by sensitized emission were in good agreement. The measurements of sensitized emission, however, involve considerable manipulation of the data (see Materials and Methods) and have a larger standard error than the donor quenching measurements. Therefore, the AO quenching data were used for quantitative comparisons of the transfer efficiency measured under different ligand conditions.

When AO was bound in the presence of either Mg + P_i or MgATP + Na, the energy-transfer efficiencies and the corresponding distances were identical (Table II). In contrast, a small but significant decrease ($P < 0.01$) in the transfer efficiency was observed when AO was bound in the presence of Mg + vanadate compared to that seen with the phosphorylating ligands (Table II). No significant differences were seen in the quantum yield of bound AO, in the shape of the AO emission or lucifer absorption spectra, nor in the anisotropies of either probe with Mg + vanadate compared to the values obtained with either Mg + P_i or MgATP + Na. Therefore, the decreased efficiency of energy transfer with Mg + vanadate suggests that the distance between AO and lucifer increased in the presence of these ligands. The estimated increase in average distance was 2 Å (Table II).

We have shown (Fortes & Lee, 1984; Lee & Fortes, 1985b) that AO can bind also to the ADP-sensitive, K-insensitive phosphoenzyme intermediate $E_1P(Na)$, which contains occluded Na (Glynn et al., 1984), and to a third phosphoenzyme form, denoted E_xP, which is partially saturated with Na at low-affinity external sites (Nørby et al., 1983; Lee & Fortes, 1985b; Yoda & Yoda, 1986). Energy-transfer measurements between AO and lucifer might detect possible conformational differences between the above enzyme forms and those obtained under the conditions of the experiments in Table II. Unfortunately, however, experimentally usable concentrations of the AO complexes with $E_1P(Na)$ and E_xP could not be obtained at equilibrium because of the antagonism between Na and AO binding (Lee & Fortes, 1985b). Oligomycin (20 μg/mL), which stabilizes $E_1P(Na)$ (Fahn et al., 1966), had no effect on the efficiency of energy transfer when AO binding was promoted by 40 mM Na + MgATP and inhibited AO binding when the Na concentration was increased above 0.4

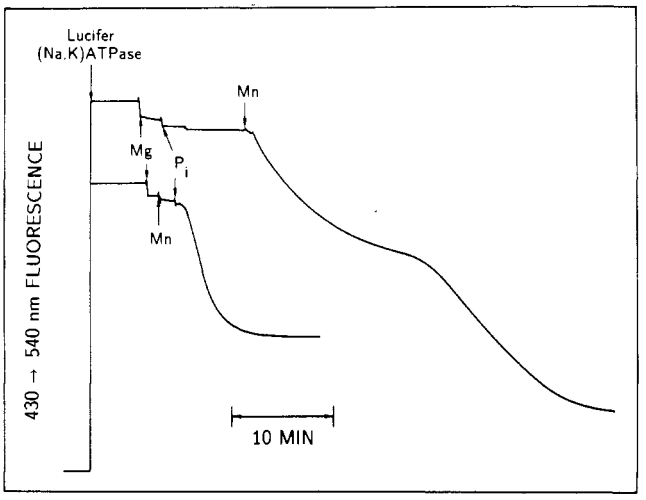

FIGURE 4: Effect of Mg, Mn, and P_i on the fluorescence of lucifer-labeled (Na,K)-ATPase. Chart traces of two experiments are shown. The following additions were made to 25 mM Tes–NH_4OH buffer (pH 6.8) at the times indicated by the arrows: 8.4 μg/mL (Na,K)-ATPase containing 0.52 μM lucifer, 10 mM $MgCl_2$, 1.1 mM $MnCl_2$, and 5 mM P_i–Tris. The temperature was 24 °C. The gain was different for the two experiments.

M (not shown). These results are consistent with the suggestion (Fortes & Lee, 1984) that bindings of oligomycin and AO are mutually exclusive.

Effect of Ligands on Fluorescence of Lucifer-Labeled (Na,K)-ATPase. We studied the effect of several ligands to investigate the possibility that changes in conformation of the β-subunit might be detected by changes in lucifer fluorescence. The following ligands, and combinations thereof, were tested: Na, K, ATP, vanadate, Mg + P_i or Na + MgATP ± ouabain, and ouabain binding to the enzyme–vanadate complex. These ligands were used in the concentration ranges that have been shown to alter proteolytic patterns (Jørgensen, 1975; Castro & Farley, 1979; Koepsell, 1979) or the fluorescence of various probes (Fortes, 1977; Karlish, 1980; Moczydlowski & Fortes, 1981a; Hegyvary & Jørgensen, 1981; Taniguchi et al., 1983; Kapakos & Steinberg, 1982; Skou & Esmann, 1981). In addition, we tested the effect of K + Mg + ATP, which alters the susceptibility of the β-subunit to proteolysis (Lo & Titus, 1978; Koepsell, 1979).

None of the above combinations of ligands caused any significant (>1%) changes in the fluorescence of lucifer-labeled (Na,K)-ATPase.

In contrast to the lack of effect of the above ligands, relatively high concentrations of $MgCl_2$ or $MnCl_2$ decreased the fluorescence of lucifer-labeled (Na,K)-ATPase (4% with 10 mM Mg, 10% with 14 mM Mn, no effect with 1 mM Mn) but did not affect the fluorescence of free lucifer in solution. These fluorescence decreases did not saturate with up to 25 mM $MgCl_2$ and were not detected at high ionic strength (buffered 2.5 M NaCl or 2.5 M tetramethylammonium chloride). Since Mn and Mg bind to catalytic sites in (Na,K)-ATPase with K_D in the 1 μM to 1 mM range (Grisham & Mildvan, 1974; Moczydlowski & Fortes, 1980), it is unlikely that the decreases in lucifer fluorescence were due to interaction of Mg or Mn with functional sites of the enzyme.

Significant changes in the fluorescence of lucifer-labeled (Na,K)-ATPase were observed after the addition of ligand combinations known to induce crystallization of (Na,K)-ATPase in the plane of the membrane (Skriver et al., 1981; Zampighi et al., 1984; Mohraz et al., 1985). Figure 4 shows two representative traces of the time course of the fluorescence changes after addition of Mn, Mg, and P_i to lucifer-labeled (Na,K)-ATPase incubated in the presence of NH_4. The large (≥50%) decreases in fluorescence required the presence of all the above ligands, regardless of their order of addition, and occurred with a lag of about 1 min after all the ligands were present (Figure 4), which suggests a cooperative process. Vanadate could substitute for P_i. When 40 μM vanadate was added instead of P_i in experiments similar to those shown in Figure 4, the fluorescence decrease was slower: 20% decrease with $t_{1/2} \sim 11$ min followed by an even slower decrease with $t_{1/2} \sim 4.5$ h (not shown). Similar, although much slower (~12 h), fluorescence decreases were observed upon incubation in the absence of Mn when the concentration of vanadate was increased to 0.7 mM (not shown). These fluorescence changes reflected perturbations of the enzyme by these ligands, because the fluorescence of free lucifer was unaffected under the above conditions.

No significant changes in fluorescence anisotropy accompanied the fluorescence changes described above. Difference absorption spectra showed that the absorbance of lucifer changed less than 6% under conditions that caused about 50% decrease in the fluorescence of lucifer-labeled (Na,K)-ATPase (incubation with 10 mM Mg + 1.1 mM Mn + 50 μM vanadate at 24 °C for 24 h). Therefore, the decreases in lucifer fluorescence, such as those shown in Figure 4, were due to an excited-state process that decreased the quantum yield of lucifer-labeled (Na,K)-ATPase.

DISCUSSION

In this work, AO and lucifer were used as probes of the cardiac glycoside site and the oligosaccharides of the β-subunit of (Na,K)-ATPase, respectively. The main findings were as follows: (1) The average distance between the cardiac glycoside site of (Na,K)-ATPase and β-subunit oligosaccharides was 47 Å. (2) This distance was the same when AO was bound with ATP or P_i as phosphorylating ligands but increased to 49 Å in the presence of vanadate. (3) Conformational changes induced by ligands were not detectable by changes in the spectroscopic parameters of lucifer attached to galactose residues of the β-subunit. (4) Ligands that cause two-dimensional crystallization of (Na,K)-ATPase quenched the fluorescence of lucifer, suggesting that crystallization involves extensive β–β interactions.

The average distance between AO and lucifer was determined from the efficiency of energy transfer measured by the decrease in the fluorescence lifetime of AO, by quenching of AO fluorescence, and by sensitized emission of lucifer (Figures 2 and 3 and Table II). The agreement between the transfer efficiencies determined by the three methods indicates that the observed energy transfer involved a homogeneous population of AO molecules adjacent to an ensemble of lucifer molecules on the β-subunit. The average distance between AO and lucifer was 47 Å, and was independent of the lucifer stoichiometry over an 11-fold range (Table I). These observations are consistent with random labeling by lucifer due to the similar reactivities of the oxidized galactose residues on the β-subunit. The average distance determined by energy transfer corresponds to the distance between the AO site and the center of the lucifer ensemble within one αβ dimer. The minimum distance between AO and the closest lucifer molecule was estimated to be 32 Å by assuming that all the observed transfer occurred between AO and a single lucifer molecule in close proximity. This is a lower limit because all the other lucifer-labeled sites would have to be located more than 81 Å from the AO site to preclude significant energy transfer from AO, which seems unlikely. Energy transfer between AO bound to one αβ dimer and lucifer attached to

the β-subunit of another $\alpha\beta$ dimer is unlikely to have been significant because the distance of closest approach between two $\alpha\beta$ dimers is about twice the R_0 value of 29 Å (Moczydlowski & Fortes, 1981c; Hebert et al., 1985; Ovchinnikov et al., 1985).

Uncertainties in the distance estimate arise mainly from errors in the orientation factor, κ^2, and in the lucifer stoichiometry. Steady-state and time-dependent anisotropy measurements showed that both AO and lucifer undergo significant motions within the lifetime of the excited state of AO. In such cases, the assumption of a random orientation between probes (i.e., $\kappa^2 = 2/3$) leads to errors of less than 10% in the estimated distance, particularly when multiple mobile acceptors are present (Dale & Eisinger, 1975; Haas et al., 1978; Stryer, 1978). The lucifer stoichiometry was determined by assuming that the protein mass of an $\alpha\beta$ dimer is 180 000 daltons. This was an overestimate, to account for the presence of contaminating peptides in the preparation. Recent data on the amino acid sequences of the α- and β-subunits indicate that the protein mass of a pure $\alpha\beta$ dimer is about 147 000 daltons (Shull et al., 1985; Kawakami et al., 1985; Noguchi et al., 1986). If the lucifer stoichiometry is calculated with the latter value, the estimated average distance between the AO site and the lucifer ensemble increases from 47 to 49 Å. Thus, the uncertainties in the estimated distance are 2–4 Å smaller than the size of the lucifer molecule and the distances between lucifer molecules on the same β-subunit.

If we assume that the average distance (47 Å) between AO and lucifer is the hypotenuse of a triangle, that the AO site is located at or near the surface of the membrane, and that the side of the triangle in the plane of the membrane has a length of 15–30 Å (Moczydlowski & Fortes, 1981c; Hall & Ruoho, 1980; Herbert et al., 1985; Ovchinnikov et al., 1985), then the distance between the center of the lucifer ensemble and the membrane surface, in the axis perpendicular to the bilayer, is 36–45 Å. This relatively long distance is consistent with the spectroscopic parameters of lucifer, which suggest that the probes attached to the β-subunit are in an aqueous environment (Lee & Fortes, 1985a). Furthermore, since the distance between the ouabain site and the ATP site or sulfhydryl groups on the α-subunit is about 70 Å (Jesaitis & Fortes, 1980; Fortes et al., 1981; Carilli et al., 1982), the overall length of (Na,K)-ATPase in the axis perpendicular to the membrane, given by the sum of these distances, is $\sim$110 Å, in agreement with the distance between ricin binding sites and the cytoplasmic edge of the protein determined by electron microscopy (Zampighi et al., 1984). On the basis of these results, and others (Moczydlowski & Fortes, 1981c), we suggest that the ATP site is at or near the cytoplasmic surface of the α-subunit.

No difference in the efficiency of energy transfer was observed when AO was bound with either Mg + P_i or MgATP + Na (Table II), suggesting that the AO–(Na,K)-ATPase complexes formed with these ligands had the same conformation. This is not unexpected, because both of these ligand combinations are thought to shift the enzyme to the same intermediate form, E_2P (Post et al., 1969; Sen et al., 1969; Yoda & Yoda, 1982). Thus, the differences in cardiac glycoside binding kinetics between the complexes formed with ATP or P_i [see Forbush (1983) and Hansen (1984) for review] may not reflect significant conformational changes, or if they do, they do not include detectable changes in the distance between the ouabain site and β-subunit oligosaccharides.

A small, but significant, decrease in the efficiency of energy transfer was observed in the presence of Mg + vanadate (Table II, donor quenching). This change in energy-transfer efficiency was not due to changes caused by Mg + vanadate in the quantum yield of AO, in the absorbance of lucifer, nor in the anisotropy of the probes. Therefore, the change reflects an increase of 2 Å in the average distance between AO and lucifer when the enzyme–AO complex was formed with vanadate compared with the phosphorylating ligands (Table II). Conformational differences between the AO complexes induced by the above ligands are not likely to involve motions larger than a few angstroms, which suggests that the small change observed does reflect a difference in conformation between these AO–(Na,K)-ATPase complexes. In addition, the labeled oligosaccharides are attached to at least two different sites on the β-subunit (Chin, 1985), so that relative motion of AO away from one of these sites could decrease the distance between AO and the other site, partially canceling the change in energy-transfer efficiency. Therefore, the 2-Å increase in distance is a low estimate of the magnitude of the difference in conformation between the AO complexes formed with vanadate and the phosphorylating ligands. It has been proposed that vanadate is a transition-state analogue of phosphate in (Na,K)-ATPase (Cantley et al., 1978) and other enzymes (Lindquist et al., 1973; Van Etten et al., 1974; Lopez et al., 1976). An interesting possibility is that the AO complex in the presence of vanadate reflects the conformation of the transition state during which the aspartyl–phosphate bond at the active site (Bastide et al., 1973) is being formed or broken, whereas in the presence of ATP or P_i the conformation of the AO complex corresponds to that of the E_2P state (Post et al., 1969; Sen et al., 1969). Then, the change in energy transfer would reflect the difference between these conformations in the plane connecting the ouabain site and the lucifer-labeled oligosaccharides.

The lack of effect on lucifer fluorescence of various ligands of (Na,K)-ATPase that alter the conformation of the α-subunit does not exclude the possibility that the β-subunit undergoes conformational changes. First, as mentioned above, the sites labeled with lucifer are about 40 Å from the membrane surface and in an aqueous environment. Conformation changes in the protein portion of the β-subunit may not be transmitted over such a distance. Second, even if they did, motions of lucifer from one aqueous environment to another would not cause a change in fluorescence. On the other hand, it is unlikely that the β-subunit plays an active role in ion pumping because other ion-transporting ATPases have similar mechanisms and sequences that are highly homologous with that of the α-subunit (MacLennan et al., 1985; Walderhaug et al., 1985; Goormaghtigh et al., 1986) but lack the equivalent of the β-subunit.

The ligand conditions that decreased the fluorescence of lucifer-labeled (Na,K)-ATPase (Figure 4) are known to induce formation of two-dimensional crystals of (Na,K)-ATPase (Skriver et al., 1981; Zampighi et al., 1984; Mohraz et al., 1985). In addition, the rapid decrease in the fluorescence of lucifer-labeled (Na,K)-ATPase in the presence of Mg + Mn + P_i correlates with the rapid formation of enzyme crystals (complete in less than 1 h) in the same medium (Zampighi et al., 1984), whereas the slow decrease in fluorescence in the presence of Mg + vanadate ± Mn correlates with the slow formation of enzyme crystals observed over a period of several days at low temperature (Skriver et al., 1981).

These results suggest that the fluorescence of lucifer-labeled oligosaccharides may be sensitive to environmental changes induced by two-dimensional crystallization of (Na,K)-ATPase, possibly through β–β interactions within the crystals. Although no attempt was made to correlate the decrease in fluorescence

with crystallization, further study of this effect may provide a spectroscopic means to monitor crystallization of (Na,K)-ATPase.

ACKNOWLEDGMENTS

We thank J. Yguerabide and E. Yguerabide for the use of the lifetime instrument and software.

Registry No. AO, 62026-02-2; ATP, 56-65-5; MgATP, 1476-84-2; ATPase, 9000-83-3; PO_4^{3-}, 14265-44-2; VO_4^{3-}, 14333-18-7; Na, 7440-23-5; Mg, 7439-95-4; Mn, 7439-96-5.

REFERENCES

Albers, R. W., Koval, G. I., & Siegel, G. I. (1968) *Mol. Pharmacol. 4*, 325–336.

Bastide, F., Meissner, G., Fleischer, S., & Post, R. L. (1983) *J. Biol. Chem. 248*, 8385–8391.

Brotherus, J. B., Møller, J. B., & Jørgensen, P. L. (1981) *Biochem. Biophys. Res. Commun. 100*, 146–154.

Cantley, L. C., Cantley, L. G., & Josephson, L. (1978) *J. Biol. Chem. 253*, 7361–7368.

Carilli, C. T., Farley, R. A., Perlman, D. M., & Cantley, L. C. (1982) *J. Biol. Chem. 257*, 5601–5606.

Castro, J., & Farley, R. A. (1979) *J. Biol. Chem. 254*, 2221–2228.

Chin, G. J. (1985) *Biochemistry 24*, 5943–5947.

Chong, P. L. G., Fortes, P. A. G., & Jameson, D. M. (1985) *J. Biol. Chem. 260*, 14484–14490.

Craig, W. S. (1982) *Biochemistry 21*, 5707–5717.

Craig, W. S., & Kyte, J. (1980) *J. Biol. Chem. 255*, 6262–6269.

Dale, R. E., & Eisinger, J. (1975) in *Biochemical Fluorescence Concepts* (Chen, R. F., & Edelhoch, H., Eds.) Vol. 1, pp 115–280, Dekker, New York.

Fahn, S., Koval, G. J., & Albers, R. W. (1966) *J. Biol. Chem. 241*, 1882–1889.

Forbush, B. (1983) *Curr. Top. Membr. Transp. 19*, 167–201.

Förster, T. (1948) *Ann. Phys. (Leipzig) 2*, 55–75.

Förster, T. (1960) *Radiat. Res., Suppl. 2*, 326–339.

Fortes, P. A. G. (1977) *Biochemistry 16*, 531–540.

Fortes, P. A. G. (1986) *Anal. Biochem. 158*, 454–462.

Fortes, P. A. G., & Lee, J. A. (1984) *J. Biol. Chem. 259*, 11176–11179.

Fortes, P. A. G., & Han, M. K. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 1443.

Fortes, P. A. G., Moczydlowski, E. G., Yagi, A., & Lee, J. A. (1981) *Abstracts of the VII International Biophysics Congress*, Mexico City, p 66.

Gennis, R. B., & Cantor, C. R. (1972) *Biochemistry 11*, 2509–2517.

Glynn, I. M. (1985) *The Enzymes of Biological Membranes* (Martonosi, A., Ed.) Vol. 3, pp 35–114, Plenum, New York.

Glynn, I. M., Hara, Y., & Richards, D. E. (1984) *J. Physiol. (London) 351*, 531–547.

Goormaghtigh, E., Chadwick, C., & Scarborough, G. A. (1986) *J. Biol. Chem. 261*, 7466–7471.

Grisham, C. M., & Mildvan, A. S. (1974) *J. Biol. Chem. 249*, 3187–3197.

Haas, E., Katchalsky-Katzir, E., & Steinberg, I. Z. (1978) *Biochemistry 17*, 5064–5070.

Hall, C., & Ruoho, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4529–4533.

Hansen, O. (1979) *Biochim. Biophys. Acta 568*, 265–269.

Hansen, O. (1984) *Pharmacol. Rev. 36*, 143–163.

Hebert, H., Skriver, E., & Maunsbach, A. B. (1985) *FEBS Lett. 187*, 182–186.

Hegyvary, C., & Jørgensen, P. L. (1981) *J. Biol. Chem. 256*, 6296–6303.

Jesaitis, A. J., & Fortes, P. A. G. (1980) *J. Biol. Chem. 255*, 459–467.

Jørgensen, P. L. (1975) *Biochim. Biophys. Acta 401*, 399–415.

Jørgensen, P. L. (1986) *Kidney Int. 29*, 10–20.

Kapakos, J. G., & Steinberg, M. (1982) *Biochim. Biophys. Acta 527*, 115–130.

Karlish, S. J. D. (1980) *J. Bioenerg. Biomembr. 12*, 111–136.

Karlish, S. J. D., & Kempner, E. S. (1984) *Biochim. Biophys. Acta 776*, 288–298.

Kawakami, K., Noguchi, S., Noda, M., Takahashi, H., Ohta, T., Kawamura, M., Nojima, H., Nagano, K., Hirose, T., Inayama, S., Hayashida, H., Miyata, T., & Numa, S. (1985) *Nature (London) 316*, 733–736.

Koepsell, H. (1979) *J. Membr. Biol. 48*, 69–94.

Kyte, J. (1971) *J. Biol. Chem. 246*, 4157–4165.

Lee, J. A., & Fortes, P. A. G. (1983) *Biophys. J. 41*, 369a.

Lee, J. A., & Fortes, P. A. G. (1985a) *Biochemistry 24*, 322–330.

Lee, J. A., & Fortes, P. A. G. (1985b) in *The Sodium Pump* (Glynn, I. M., & Ellory, J. C., Eds.) pp 278–282, The Company of Biologists, Cambridge.

Lindquist, R. N., Lynn, J. L., Jr., & Lienhard, G. E. (1973) *J. Am. Chem. Soc. 95*, 8762–8768.

Lo, T. N., & Titus, E. O. (1978) *J. Biol. Chem. 253*, 4432–4438.

Lopez, V., Stevens, T., & Lindquist, R. N. (1976) *Arch. Biochem. Biophys. 175*, 31–38.

MacLennan, D. H., Brandl, C. J., Korczak, B., & Green, N. M. (1985) *Nature (London) 316*, 696–700.

Matsui, H., & Schwartz, A. (1968) *Biochim. Biophys. Acta 151*, 655–663.

Moczydlowski, E. G., & Fortes, P. A. G. (1980) *Biochemistry 19*, 969–977.

Moczydlowski, E. G., & Fortes, P. A. G. (1981a) *J. Biol. Chem. 256*, 2346–2356.

Moczydlowski, E. G., & Fortes, P. A. G. (1981b) *J. Biol. Chem. 256*, 2357–2366.

Moczydlowski, E. G., & Fortes, P. A. G. (1981c) *Biophys. J. 33*, 132a.

Mohraz, M., Yee, M., & Smith, P. R. (1985) *J. Ultrastruct. Res. 93*, 17–26.

Noguchi, S., Noda, M., Takahashi, H., Kawakami, K., Ohta, T., Nagano, K., Hirose, T., Inayama, S., Kawamura, M., & Numa, S. (1986) *FEBS Lett. 196*, 315–320.

Nørby, J. G., Klodos, I., & Christiansen, N. O. (1983) *J. Gen. Physiol. 82*, 725–759.

Ovchinnikov, Y. A., Demin, V. V., Barnakov, A. N., Kuzin, A. P., Lunev, A. V., Modyanov, N. N., & Dzhandzhugazyan, K. N. (1985) *FEBS Lett. 190*, 73–76.

Post, R. L., Kume, S., Tobin, T., Orcutt, B., & Sen, A. K. (1969) *J. Gen. Physiol. 54*, 306s–326s.

Ruoho, A., & Kyte, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2352–2356.

Schwartz, A., Matsui, H., & Laughter, A. H. (1968) *Science (Washington, D.C.) 160*, 323–325.

Sen, A. K., Tobin, T., & Post, R. L. (1969) *J. Biol. Chem. 244*, 6596–6604.

Shinitzky, M. (1972) *J. Chem. Phys. 56*, 5979–5981.

Shull, G., Schwartz, A., & Lingrel, J. B. (1985) *Nature (London) 316*, 691–695.

Skou, J. C., & Esmann, M. (1981) *Biochim. Biophys. Acta 647*, 232–240.

Skriver, E., Maunsbach, A. B., & Jørgensen, P. L. (1981) *FEBS Lett. 131*, 219–222.
Stryer, L. (1965) *J. Mol. Biol. 13*, 482–495.
Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819–846.
Taniguichi, K., Suzuki, K., & Iida, S. (1983) *J. Biol. Chem. 258*, 6927–6931.
Van Etten, R. L., Waymack, P. P., & Rehkop, D. M. (1974) *J. Am. Chem. Soc. 96*, 6782–6785.
Walderhaug, M. O., Post, R. L., Saccomani, G., Leonard, R. T., & Briskin, L. P. (1985) *J. Biol. Chem. 260*, 3852–3859.
Wallick, E. T., Lindenmayer, G. E., Lane, L. K., Allen, J. C., Pitts, B. J. R., & Schwartz, A. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 2214–2218.
Wallick, G. T., Lane, L. K., & Schwartz, A. (1979) *J. Biol. Chem. 254*, 8107–8109.
Yguerabide, J. (1972) *Methods Enzymol. 26*, 498–578.
Yoda, A., & Yoda, S. (1982) *Mol. Pharmacol. 22*, 693–699.
Yoda, A., & Yoda, S. (1986) *J. Biol. Chem. 261*, 1147–1152.
Zampighi, G., Kyte, J., & Freytag, W. (1984) *J. Cell Biol. 98*, 1851–1864.

Binding and Hydrolysis of ATP by Cardiac Myosin Subfragment 1: Effect of Solution Parameters on Transient Kinetics†

Jo H. Hazzard and Michael A. Cusanovich*
Department of Biochemistry, University of Arizona, Tucson, Arizona 85721
Received April 21, 1986; Revised Manuscript Received September 3, 1986

ABSTRACT: Transient kinetic data of ATP binding and cleavage by cardiac myosin subfragment 1 (S1) were obtained by fluorescence stopped flow and analyzed by using computer modeling based on a consecutive, reversible two-step mechanism:

$$M_0 + ATP \underset{k_{21}}{\overset{K'_0 k_{12}}{\rightleftharpoons}} M_1{\cdot}ATP \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} M_{12}{\cdot}ADP{\cdot}P_i$$

where M_1 and M_{12} denote myosin species with enhanced fluorescence and $K'_0 = K_0/(K_0[ATP] + 1)$. The kinetic constants K_0, k_{12}, k_{23}, and k_{32} and the fractional contributions of M_1 and M_{12} to the total fluorescence are analyzed over a range of systematically varied solution parameters. The initial ATP binding equilibrium (K_0), which decreases with increasing pH, is facilitated by a positively charged protein residue with a pK of 7.1. An active-site charge of +1.5 is determined from the ionic strength dependence. The rate constants k_{12}, k_{23}, and k_{32} also exhibit pK's near neutrality but increase with increasing pH. The majority of the large (−54 kJ/mol) negative free energy of ATP binding occurs upon S1 isomerization, k_{12}, and a large increase in entropy (183 J/kmol at 15 °C) is associated with the cleavage step. The equilibrium constant for the cleavage step, K_2, is determined as 3.5 at pH 7.0, 15 °C, and 200 mM ionic strength. There are no significant changes in fractional contributions to total fluorescence enhancement due to solvent-dependent conformational changes of S1 in these data. When values for the combined rate constants are calculated and compared with those determined by graphical analysis, it is observed that graphical analysis overestimates the binding rate constant (K_0k_{12}) by 25% and the hydrolysis rate constant ($k_{23} + k_{32}$) by as much as 30%. Comparison of these data for cardiac S1 with available data for skeletal S1 indicates that the proteins have similar combined rate constants except for K_0k_{12}, which is approximately 7 times larger for skeletal S1. As the two proteins' electrostatic dependencies are similar, a steric constraint to nucleotide binding in the cardiac S1 relative to the skeletal S1 is postulated.

The mechanism of ATP binding and hydrolysis by myosin and its catalytic subfragment 1 (S1)[1] consists of at least three steps: (a) formation of a collision complex in rapid equilibrium with reactants followed by (b) an essentially irreversible conformational change resulting in an enhancement of fluorescence and (c) the rate-limiting step in which the γ-phosphate bond is cleaved, resulting in an additional fluorescence enhancement (Bagshaw & Trentham, 1974; Johnson & Taylor, 1978). This is given by the expression:

$$M_0 + T \underset{}{\overset{K_0}{\rightleftharpoons}} M_0{\cdot}T \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} M_1{\cdot}T \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} M_{12}{\cdot}D{\cdot}P_i \quad (1)$$

where M_0 is myosin S1, M_1 and M_{12} denote S1 with enhanced fluorescence, and T, D, and P_i refer to ATP, ADP, and inorganic phosphate, respectively. The product release steps can be considered separately from binding and hydrolysis as the first step in eq 2 is the overall rate-limiting step (Bagshaw &

$$M_{12}{\cdot}D{\cdot}P_i \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} M_{12}{\cdot}D + P_i \overset{K_4}{\rightleftharpoons} M_1{\cdot}D \overset{K_5}{\rightleftharpoons} M_0{\cdot}D \overset{K_6}{\rightleftharpoons} M_0 + D \quad (2)$$

Trentham, 1974; Johnson & Taylor, 1978) and is quite slow

† This research was supported in part by U.S. Public Health Service Grant HL 28906 to M.A.C. and NIH Postdoctoral Fellowship HL 07107 and American Heart Association, Arizona Affiliate, Grant-in-Aid to J.H.H.

[1] Abbreviations: S1, myosin subfragment 1; BTP, 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane; DTT, dithiothreitol; HMM, heavy meromyosin; MES, 2-(*N*-morpholino)ethanesulfonic acid; HPLC, high-performance liquid chromatography.

0006-2960/86/0425-8141$01.50/0