Immunochemical and Spectroscopic Characterization of Two Fluorescein 5′-Isothiocyanate Labeling Sites on Na^+,K^+-ATPase†

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ABSTRACT: Fluorescein 5′-isothiocyanate (FITC) covalently modifies the Lys-501 residue of the catalytic (α) subunit of Na^+,K^+-ATPase and resides at a conformation-sensitive site in or near the ATP binding site. In these studies, FITC-directed antibodies which quench this hapten's fluorescence were used to infer the solvent accessibility of the enzyme-bound probe. These antibodies identified two FITC labeling populations. An antibody-accessible population, representing 20–50% of the bound FITC fluorescence, was essentially (95%) quenched by the antibody. The second population was irreversibly labeled, was inaccessible to antibody, and was the fraction of probe whose fluorescence intensity is sensitive to the enzyme's conformation. The anti-FITC antibodies therefore permitted the selective investigation of FITC at this active site. Distinct differences between the two labeling sites were then demonstrated. Shifts in the absorption spectrum suggested that the active-site-bound probe resides in a hydrophobic environment, while polarization values indicated a rigid, rotationally restricted location. These two properties were not altered by ligand additions. Iodide quenching studies, however, showed that in the E_1Na^+ conformation there was a 50% decrease in solvent access to the active-site-bound probe as compared to free probe while the $E_1Na^+ \rightarrow E_2K^+$ transition decreased this accessibility an additional 50%. Similarly, there was a significant decrease in the relative quantum yield of FITC linked at this site that was reduced further by the $E_1Na^+ \rightarrow E_2K^+$ transition. In contrast, frequency domain spectroscopy showed no significant differences in the lifetimes of fluorescence decay for the two different labeling populations nor for the high (E_1Na^+) and low (E_2K^+) fluorescence intensity conformations. We have found that static (lifetime independent) quenching rather than collisional processes or protonation changes accounts for the fluorescence intensity changes undergone by FITC bound at the ATP-protectable site.

The sodium and potassium pump (Na^+,K^+-ATPase, EC 3.6.1.3) of the plasma membrane of animal cells has been extensively studied since it was first identified in crab nerve membranes by Skou (1957). This enzyme, composed of a catalytic (α) and a glycoprotein (β) subunit, is of considerable importance not only because it serves a fundamental role in preserving cellular homeostasis but also because it is the receptor for the cardiotonic drugs, the cardiac glycosides (Schatzmann, 1953).

Several lines of evidence have contributed to our current understanding of the molecular mechanisms of the ATP-dependent ion transport by this enzyme. Kinetic studies have distinguished a minimum of four conformations that contribute to the reaction cycle: the E_1Na^+ and E_2K^+ forms and the phosphoenzyme intermediates, $E_1 \sim P$ and E_2–P (Albers, 1967; Post et al., 1969). Recently the complete primary structure of α has been deduced from its cDNA sequences (Shull et al., 1985; Kawakami et al., 1985), and this has allowed initial predictions of its organization in the membrane. In addition, the determination of the sites of chemical labeling of α by fluorescent and photoaffinity probes and the effects of site-directed mutagenic changes have made it possible to begin identifying specific primary sequence regions which may contribute to the ATP, cation, and cardiac glycoside binding domains (Jorgensen & Anderson, 1988; Glynn, 1985; Ovchinnikov, 1987; Price & Lingrel, 1988). Fluorescence energy-transfer measurements made between fluorescent probes have also indicated the transmembrane distances from the cardiac glycoside binding site to the ATP site and the lateral distance to the β subunit (Carilli et al., 1982; Lee & Fortes, 1985).

The fluorescein derivative fluorescein 5′-isothiocyanate (FITC) has proven to be a useful fluorescent probe for studies of the Na^+,K^+-ATPase. It has been used extensively because it undergoes reversible fluorescence intensity changes that correlate with ligand-induced alterations undergone by the enzyme (Karlish, 1980; Hegyvary & Jorgensen, 1981; Davis & Robinson, 1988; Friedman & Ball, 1989). It has been shown to specifically label the lamb kidney enzyme at the Lys-501 of α (Farley et al., 1984). This site is located on the cytoplasmic side of the enzyme and resides within a sequence region highly conserved among ion transport ATPases (Kirley et al., 1984; Shull et al., 1985; Kyte et al., 1987). The fact that FITC labeling of α inactivates the enzyme and ATP can prevent both the labeling and inactivation has led to the general assumption that this sequence region is part of the ATP binding site. It is possible though that it is the positioning of the xanthone ring of FITC in or near a portion of the adenine nucleotide binding site rather than the modification of Lys-501 that explains the activity inhibition (Ball & Friedman, 1987). For example, changing the equivalent Lys-515 to alanine in the Ca^{2+}-ATPase of the sarcoplasmic reticulum alters enzyme activity but does not appear to affect ATP binding to or phosphorylation of the enzyme (Maruyama et al., 1989).

In addition, while fluorescein has dimensions similar to the adenine moiety, in the presence of K^+ the FITC-labeled enzyme can still hydrolyze small phosphorylated compounds (i.e., *p*-nitrophenyl phosphate) and the larger 3-*O*-methylfluorescein

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phosphate (Davis & Robinson, 1988). This suggests that under E_2K^+ conditions the covalently linked FITC molecule may move sufficiently to allow the "low affinity" ATP site to be available for either nucleotide or 3-*O*-methylfluorescein binding (Davis & Robinson, 1988).

Clearly, a more thorough understanding of the physical interactions between α and FITC is needed in order to determine what aspects of the protein's conformation changes FITC actually reports. Therefore, we have investigated the protein and solvent environment about the probe as indicated by its fluorescence lifetime, polarization, and quenching properties. Despite the extent to which this probe has been used, little attention has been focused upon how its fluorescent properties are altered.

In this study, we have used fluorescein-directed antibodies to demonstrate the presence of at least two different populations of enzyme-bound FITC. Using standard labeling techniques (Farley et al., 1984; Kirley et al., 1984), we have found a reversibly bound, antibody-accessible, fraction that can be quenched by antibody binding and which accounts for 20–50% of the total enzyme-bound probe detected by fluorescence spectroscopy. The second population is inaccessible to antibody and has been found to account for the fluorescence intensity changes that are observed upon ligand additions. Therefore, the extent of the changes in fluorescence intensity undergone by the probe linked to Lys-501 is likely to have generally been underestimated. Measurements of fluorescence decay lifetimes did not distinguish between the two different labeling sites, but collisional quenching constants obtained from iodide quenching data demonstrated that the sequestered probe has a significantly reduced solvent accessibility compared to that of either free or enzyme-bound, antibody-accessible FITC. Solvent accessibility of this probe population was also reduced by the $E_1Na^+ \rightarrow E_2K^+$ transition. In contrast, the rotational motions of the Lys-501-linked probe, as measured by anisotropy determinations, are highly restricted but not altered by different ligand conditions. The demonstration of two different labeling populations of FITC suggests that calculations of distances between the FITC site and other intracellular sites on α as based upon Föster-type fluorescence energy transfer may need further investigation. The distance to the anthroylouabain binding site, however, was unchanged. Our studies and the recent work of Xu (1989) reporting that FITC may label other residues of α in addition to Lys-501 add another level of complexity to the interpretations of previous studies.

Materials and Methods

Na^+,K^+-ATPase Isolation. The Na^+,K^+-ATPase was purified from the outer medulla of frozen lamb kidneys by the method of Lane et al. (1979) and stored at 4 °C until used. After polyacrylamide gel electrophoresis (PAGE), the protein showed only two protein bands corresponding to the molecular weights of the α (~100K) and β (~50K) subunits. The initial specific activity when purified was between 1000 and 1200 μmol of P_i mg^{-1} h^{-1}. Activity measurements were made as described by Ball and Friedman (1987).

FITC Labeling of the Na^+,K^+-ATPase. Labeling of the enzyme with 5–10 μM FITC was accomplished by adding 1 mM FITC, freshly dissolved in DMSO, to Na^+,K^+-ATPase (1 mg/mL) in 100 mM Tris-HCl, pH 9.1, for 30 min in the dark at 23 °C according to Kirley et al. (1984). For labeling over the 0–30 μM FITC range, the stock FITC solution was 0.1 mM in 10% DMSO which was added to the protein at ratios ranging from 1:100 to 3:10 (v/v). FITC labeling in the presence of ATP was done similarly with 5 mM Na_2ATP present. Unless stated otherwise, labeled enzyme was separated from unreacted probe by centrifugation (100000*g*) and resuspension 3 times or by centrifugation of the sample (100*g*) twice through a volume of G-50 Sephadex that was 3–5-fold greater than the sample volume.

Steady-State Fluorescence and Absorbance Measurements. Steady-state fluorescence measurements were made by using an SLM/AMINCO SPF-500 spectrofluorometer. FITC-labeled protein (5–20 μg) was suspended in 2.5 mL of 0.05 M Tris-HCl/1 mM EGTA buffer, pH 7.4, under continuous stirring at 25 °C. Samples were excited at 495 nm (4-nm band-pass width), and emission was recorded at 520 nm (10-nm band-pass width). For polarization determinations, the excitation band-pass width was 10 nm. Additions to the enzyme were made from stock solutions, e.g., 2 M NaCl, 2 M KCl, 1 mg/mL anti-FITC antibody, or 8 M choline iodide, to give the final concentrations as indicated for specific experiments. Absorbance measurements of FITC-labeled enzyme were made with a UVIKON 930 spectrophotometer over the spectra range 300–600 nm with unlabeled enzyme serving as a control for sample turbidity. The absorbance maximum of all spectra was less than 0.5.

Analysis of Iodide Quenching Data. Iodide has previously been shown to collisionally quench both tryptophan and fluorescein fluorescence (Eftink & Ghiron, 1981; Highsmith, 1986), and it was therefore assumed to be a collisional quencher of the enzyme-bound FITC. Fluorescence decreases were measured over a 0–100 mM choline iodide concentration since above this concentration enzymatic activity was observed to decrease. All titrations were performed in triplicate and plotted by using the Stern–Volmer equation:

$$F_0/F = 1 + K_q[Q] \quad (1)$$

where F_0 and F are the initial fluorescence intensity and the intensity in the presence of quencher [Q], respectively. K_q is the dynamic Stern–Volmer quenching constant. The quenching constant was obtained from the slope of the line of F_0/F versus [Q] as calculated by the least-squares best fit of the quenching data. In every case, the correlation coefficient for the fit of the data to a straight line was ≥ 0.997. In general, FITC labeled two populations of sites, and each could be selectively eliminated, allowing the other population to be observed and analyzed according to eq 1. The site 1 (reversibly bound) fluorescence was quenched by the addition of anti-FITC antibodies, while site 2 (ATP site) labeling was blocked by the presence of 5 mM ATP.

The fully labeled enzyme was also analyzed according to eq 1. In that case, the overall apparent quenching constant (K_{q12}) was assumed to be a linear combination of the quenching constant for each site:

$$K_{q12} = f_1K_{q1} + f_2K_{q2} \quad (2)$$

Here f_1 and f_2 are the fractions of the total fluorescence contributed from sites 1 and 2, respectively, while K_{q1} and K_{q2} are the quenching constants for sites 1 and 2, respectively, as determined individually. The fraction of fluorescence quenched by the antibody (f_1) was 0.41 and 0.49 for the E_1Na^+ and E_2K^+ conformations, respectively, while the remaining fractions were f_2. The f_1 and f_2 values varied about 3% over the iodide concentration range used. This amount of uncertainty, though, had little effect on the calculated value of K_{q12} and was therefore considered to be negligible.

Equation 2 can be rearranged to calculate K_{q1} in terms of K_{q12}, f_1, f_2, and K_{q2}:

$$K_{q1}(\text{calcd}) = (K_{q12} - f_2K_{q2})/f_1 \quad (3)$$

or similarly to calculate K_{q2}

$$K_{q2}(\text{calcd}) = (K_{q12} - f_1 K_{q1})/f_2 \quad (4)$$

Calculated values of K_{q1} and K_{q2} were then dependent upon the quenching constant K_{q12} for the fully labeled protein and the separately obtained K_q values. These were compared to the values measured when these sites were isolated by antibody quenching or ATP protection in order to determine if these procedures altered the quenching constants of these sites.

Fluorescence Lifetime Measurements. Fluorescence decay lifetimes of the enzyme-linked FITC were measured by using the phase and modulation technique (Spencer & Weber, 1969). Phase shift and demodulation measurements were made with an SLM 4800 fluorometer modified with a Pockel cell modulator as described previously by McLean et al. (1989). The excitation monochromator wavelength (bandwidth 16 nm) was 485 nm while the emission was observed through an Ealing 35-3599 interference filter (center 520 nm, bandwidth 7 nm). Modulation frequencies were varied over a 10–100-MHz range, and the phase shift (ϕ_ω) and demodulation (m_ω) values at each measured frequency, ω, were calculated relative to a glycogen standard (τ = 0 ns). Lifetime analysis was performed by using the method of Lakowicz et al. (1984). This assumes that the fluorescence intensity of each fluorescent species decays exponentially with time. The observed intensity $I(t)$ decays then as the sum of all fluorescence species:

$$I(t) = \sum_{i=1}^{N} \alpha_i e^{-t/\tau_i} \quad (5)$$

Here N is the number of components, with α_i and τ_i the preexponential factor and decay time for the ith component, respectively. The fractional contribution to the steady-state fluorescence (I) of the ith component is $\alpha_i\tau_i/\sum\alpha_i\tau_i$. The experimental values of ϕ_ω and m_ω were fit to the values calculated by using the sine and cosine transforms of eq 5 by nonlinear least squares [see Lakowicz et al. (1984) for details].

The calculated values of lifetimes and preexponential factors were those which minimized the goodness of fit parameter, X^2:

$$X^2 = \sum_\omega [(1/\delta^2_{\phi\omega})(\phi_\omega - \phi_{c\omega})^2] + \sum_\omega [(1/\delta^2_{m\omega})(m_\omega - m_{c\omega})^2] \quad (6)$$

where $\delta_{\phi\omega}$ and $\delta_{m\omega}$ are the frequency-dependent errors in the measurements of ϕ_ω and m_ω, and the calculated values of phase and modulation are $\phi_{c\omega}$ and $m_{c\omega}$. The validity of different calculated lifetime distributions (i.e., one, two, or three components) was judged by the values of the reduced, X^2_R:

$$X^2_R = (1/2N - P)X^2 \quad (7)$$

where N equals the number of frequencies used and P is the number of free parameters in the given distribution. In this study, a fixed lifetime (0.01 ns) component was included in the calculation to account for background light scattering from the turbid enzyme samples. Unlabeled enzyme showed a similar fast lifetime while the free probe had no significant second fluorescence component. For all labeled protein, the calculated fast component was a minor fraction of the total fluorescence and was the greatest in the sample with the lowest FITC fluorescence intensity (E_2K^+ + anti-FITC antibodies; Table III).

Anisotropy Measurements. Steady-state anisotropy values were calculated from polarization measurements using an SLM/AMINCO 500C fluorometer. The fluorescence intensity (I) of enzyme-bound FITC was observed in the vertical (I_{VV}) and horizontal (I_{VH}) positions when excited by vertically polarized light. Anisotropy (r), the vertically polarized light intensity relative to the total intensity, is

$$r = [(I_{VV}/I_{VH})G - 1]/[(I_{VV}/I_{VH})G + 2] \quad (8)$$

where G, an instrumentation correction made for unequal detection of vertically versus horizontally polarized light (in our case 1.3), was equal to the horizontal polarized emission divided by the vertical when the excitation polarizer was oriented horizontally. In addition, all I_{VV} and I_{VH} values were corrected for base-line contributions. In instances where the fluorescence was partially quenched, the anisotropy of the quenched population was calculated by using the equation: $r = r_1f_1 + r_2f_2$ where r is the anisotropy of the total fluorescence, r_1 and r_2 are the anisotropies of the antibody-quenched and unquenched probe populations, respectively, and f_1 and f_2 are the fractional fluorescence intensities of each population. The values of r, r_2, f_2, and f_1 were measured (see text) and used to calculate r_1.

Calculation of Limiting Anistropy (r_∞) and the Rotational Correlation Time (ϕ). To calculate the motional properties of FITC on the protein, the decay of fluorescence anisotropy was assessed by using a form of the Perrin equation modified for hindered rotations as described by Lakowicz et al. (1979):

$$r = r_\infty + (r_0 - r)/(\tau/\phi) \quad (9)$$

where ϕ is the rotational correlation time of the probe, τ is the lifetime of fluorescence, and r_0 is the anisotropy in the absence of rotational diffusion while r_∞ is the limiting anisotropy at long times ($t \rightarrow \infty$) and r is the measured anisotropy. The fluorescence lifetime was then varied by increasing concentrations of the quencher iodide [Q] while keeping a constant ionic strength with choline chloride. Iodide was assumed to behave as a collisional quencher, and the calculated lifetime was obtained by multiplying the initial unquenched lifetime (see Table III) by the relative fraction of fluorescence intensity remaining after addition of the quencher. The value of r_0 used in eq 9 was 0.390. This value was derived by taking r_0 = 0.375 as obtained by Lakowicz et al. (1985) for λ_{ex} = 442 nm and correcting it for λ_{ex} = 495 nm as used in our studies. This increase in r_0 can be seen from the anisotropy excitation spectrum of fluorescein (Jameson, 1984). The determined values of r at increasing [Q] were plotted versus $(r_0 - r)/\tau$, and r_∞ was taken from the y intercept and ϕ from the slope of the line as fitted to the data. The ratio r_∞/r_0 is taken as a measure of the average limiting angle of hindered motion for FITC at the "ATP" labeling site and is described in the text.

Gel Electrophoresis and Western Blot Analysis. SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) was performed by using a Bio-Rad minigel apparatus. Fluorescein fluorescence was detected by using a transilluminator UV (365 nm) light box. For Western blot analysis, the FITC-labeled enzyme, resolved by PAGE, was electroblotted onto nitrocellulose sheets, using a Hoefer Scientific Instruments electrophoresis unit, and individual lanes were exposed to rabbit polyclonal antiserum raised to native Na^+,K^+-ATPase or fluorescein-specific polyclonal antibodies. Enzyme or probe-bound antibody was then detected by using peroxidase-conjugated goat anti-rabbit immunoglobulin.

Other Materials. Buffers and chemical reagents were obtained from the Sigma Chemical Co. The FITC isomer I was obtained from Molecular Probes (Eugene, OR) while the anti-FITC antibodies were polyclonal fluorescein-directed antibodies generously supplied by Dr. Edward W. Voss, Jr. (University of Illinois, Urbana, IL). The immunological

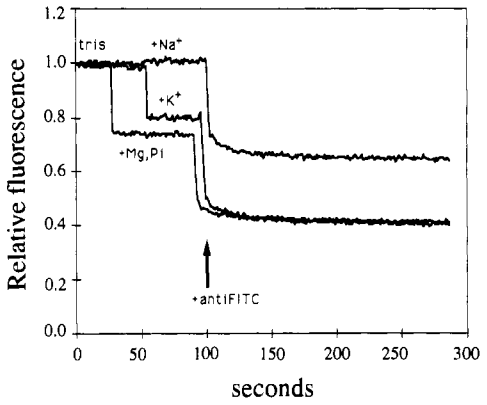

FIGURE 1: Fluorescence quenching of Na^+,K^+-ATPase-linked FITC by anti-FITC antibodies. Fifteen micrograms of FITC-Na^+,K^+-ATPase was suspended in 2.5 mL of Tris buffer, and ions as indicated were added to induce specific conformations: 10 mM NaCl for the E_1 conformation (upper curve); 10 mM KCl for the E_2 conformation (middle curve); 5 mM HPO_4–Tris + 10 mM $MgCl_2$ for the E_2–P_iMg^{2+} conformation (lower curve). The final addition to each sample was 10 μg of anti-FITC antibody. Fluorescence intensities were normalized to the values of the initial E_1 Tris conformation for each sample.

Table I: Effect of Anti-FITC Antibodies on Equilibrium Constants for Ion-Induced Conformational Changes[a]

	ligand			
	NaCl		KCl	
sample	K_d (mM)	n_H	K_d (mM)	n_H
FITC-Na^+,K^+-ATPase	11.0 ± 1.0	1.8 ± 0.3	0.25 ± 0.03	1.3 ± 0.1
FITC-Na^+,K^+-ATPase + anti-FITC Ab	11.0 ± 1.0	2.0 ± 0.1	0.35 ± 0.07	1.5 ± 0.2

[a] The E_2 to E_1 conformational transition of FITC-labeled Na^+,K^+-ATPase was monitored by the fluorescence increase when the enzyme in 2.4 mM KCl was titrated with NaCl from 0 to 100 mM. Conversely, the E_1 to E_2 conformational transition was monitored by titration with 0–10 mM KCl of the labeled enzyme in the presence of 4 mM NaCl. Data were analyzed by the Hill plot: log (fractional fluorescence change)/(1 − fractional fluorescence change)] = n_H log [ion] − n_H log K_d. Titrations were done at seven concentrations, and the linear portion of the plots through the region where [ion] $\simeq K_d$ was fit to the Hill equation. The results from triplicate data sets were averaged.

reagents were purchased from CalBiochem (La Jolla, CA).

Results

FITC Labeling and Inactivation of Na^+,K^+-ATPase. The purified lamb kidney Na^+,K^+-ATPase (1 mg/mL) labeled with 5–10 μM FITC for 30 min resulted in 90% inactivation of enzyme ATPase activity. The labeling stoichiometry determined by FITC absorbance under denaturing conditions was 2.6 ± 0.3 nmol/mg of protein (0.39 mol of FITC/mol of protein) which was similar to previous studies (Carilli et al., 1982; Kirley et al., 1984). The fluorescence intensity of the FITC-labeled enzyme was also sensitive to enzyme conformation. The E_1 conformation, induced by addition of Na^+ ions, had a 20–25% higher fluorescence intensity than the E_2 forms induced by K^+ or by Mg^{2+}, P_i, respectively (Figure 1). The change in FITC fluorescence upon ion addition was used to measure the K_d and Hill coefficients for the Na^+- and K^+-induced transitions (Table I) with results consistent with the original report by Karlish (1980) and our more recent work (Friedman & Ball, 1989).

Binding of Anti-FITC Antibodies. The binding of affinity-purified anti-fluorescein antibodies to the enzyme-linked probe was then investigated in order to determine the relative

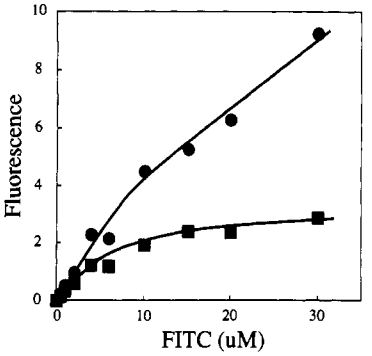

FIGURE 2: Demonstration of antibody-accessible and -inaccessible FITC populations on Na^{2+},K^+-ATPase. Na^+,K^+-ATPase (1 mg/mL) was labeled at varying FITC concentrations, and the total fluorescence intensity of 5 μg of labeled enzyme in 2.5 mL of Tris buffer is shown by the closed circle curve (●). The antibody-inaccessible fluorescence was then quantitated by the addition of excess anti-FITC antibody (15 μg) (■). Data are from a single experiment that has been reproduced with similar results with labeling done at 0.5 and 0.3 mg/mL Na^+,K^+-ATPase.

exposure or accessibility of FITC at its labeling sites. In control experiments, the antibody quenched 95% of the fluorescence of fluorescein and FITC free in solution with a $K_d < 1$ nM (data not shown), which is consistent with previous work (Watt & Voss, 1978). The quenching of enzyme-bound FITC fluorescence was therefore taken as an indication of antibody binding.

The effect of anti-FITC antibodies on enzyme-bound FITC fluorescence is shown in Figure 1. When saturating antibody (10 μg) was added, a partial quenching of FITC fluorescence was observed for the enzyme (15 μg) in the following conformations: E_1Na^+, E_2K^+, and E_2–P_iMg^{2+}. Characteristically, 20–50% of the original fluorescence intensity was quenched within 10 s (40% quenching in Figure 1) of antibody addition, and an additional 10% was quenched with a half-life of 5–10 min. Figure 1 also shows that the extent of FITC quenching was almost identical for each enzyme conformation, suggesting that the antibody-accessible fraction was a distinct, conformation-independent, fraction of the bound probe. This was supported by the subsequent experiment that showed the same absolute levels for the ion-induced fluorescence intensity changes when the antibody quenching of FITC was initiated before ligand addition (data not shown). Thus, two main populations of labeled sites were identified by the antibody quenching and by ligand-induced conformational enhancement or quenching. As shown in Table I, the antibody quenching of FITC did not affect the K_D values for Na^+ and K^+ binding or their cooperative interactions as monitored by the intensity changes of the remaining FITC fraction.

With the antibody present, the relative quenching by the E_1Na^+ to E_2K^+ conformation transition was considerably increased, rising from the usually reported 20% change (Karlish, 1980; Hegyvary & Jorgensen, 1981) to a 35–45% change, suggesting that previous studies have also had these two labeling populations.

Relative Stoichiometry of FITC Labeling of Na^+,K^+-ATPase. In order to investigate the relative stoichiometries of the FITC labeling sites, the enzyme was labeled with FITC over a concentration range of 0–30 μM. Figure 2 shows the total fluorescence intensity of bound probe and the amount of fluorescence remaining after antibody quenching. The difference between these curves represented the antibody-quenched fluorescence. The antibody-inaccessible labeling population was saturated over the FITC concentration range 5–10 μM. All subsequent labeling occurred at antibody-ac-

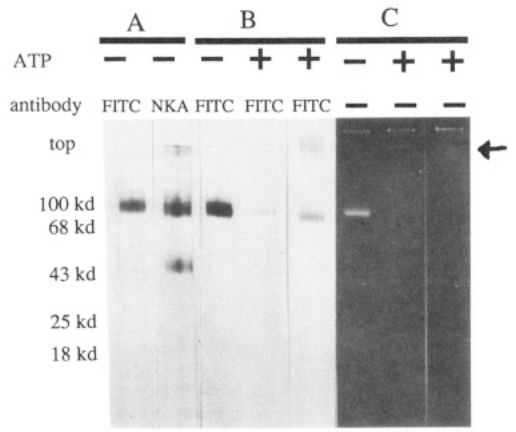

FIGURE 3: Detection of FITC labeling of Na^+,K^+-ATPase by UV fluorescence and Western blot analysis. In gel A, labeled enzyme (0.5 μg per lane) was detected by FITC-specific antibodies (first lane) and by Na^+,K^+-ATPase-specific antibodies (second lane). In gel B, FITC-specific antibodies detected 0.5 μg of FITC–enzyme but not 0.5 μg of enzyme labeled in the presence of 5 mM ATP (second lane) and only slightly stained 5 μg of the +ATP sample (gel B, third lane). In gel C, 5 μg of FITC–enzyme showed the α-subunit fluorescence (first lane) but none for 5 μg of the +ATP sample (second lane). The last lane on gel C showed a diffuse fluorescent band (at the arrow) for 5 μg of the +ATP sample when the gel was run for 5 min and the dye front advanced 1.5 cm.

cessible sites. The lack of saturation and relatively high fluorescence for the latter fraction initially suggested that this binding was of low-affinity ($K_d \geq 30$ μM) nonspecific labeling that occurred at more than one site.

However, the relative proportions for the two FITC populations were comparable throughout the initial 1–10 μM FITC concentration range. This suggested that there was no discernible difference in the inherent reactivities of the two sites. Further, after addition of anti-FITC antibody, the relative decrease in fluorescence with the $E_1Na^+ \rightarrow E_2K^+$ transition was approximately constant (39 ± 2%) for all levels of labeling, for FITC concentrations of 1–30 μM. This suggested that the titration of a single class of labeling site was monitored in the presence of the antibody.

Characterization of the Labile, or Nonspecific, FITC Labeling. Our data suggested that the antibodies bound to a probe population that had not been accounted for or detected in previous sequencing work nor by PAGE resolution of enzyme labeled with ATP present (Karlish, 1980). To investigate this, Na^+,K^+-ATPase was labeled with 10 μM FITC in the presence and absence of 5 mM ATP. After removal of unbound FITC, enzyme labeled in the presence of ATP was found to have about half the FITC fluorescence of enzyme labeled in the absence of ATP, and this fluorescence was nearly completely (95%) quenched by the addition of anti-FITC antibodies.

As reported by Karlish (1980), we also found PAGE resolved α-subunit highly fluorescent for the fully labeled sample but no fluorescence was detectable in the gel for the sample labeled in the presence of ATP (Figure 3, gel C). Western blot analysis of the enzyme using the fluorescein-specific antibodies was also used to detect FITC-labeled α whose fluorescence may have been quenched within the gel. The anti-FITC antibody was found to bind to the minus ATP sample at least 10-fold more than the plus ATP sample (Figure 3, gel B). The apparent lack of labeling of α by FITC with ATP present was further analyzed by running enzyme samples on the acrylamide gels for different lengths of time. When the sample was run for 5 min, a diffuse fluorescent band 0.5-cm wide appeared slightly ahead of the protein but well behind the dye front (arrow in Figure 3, gel C). As the gel was run for 10 and then 30 min, this fluorescent band became more diffuse until it was undetectable. This fluorescence was not associated with any Coomassie blue stained protein band and yet ran behind the dye front and free FITC (data not shown). Therefore, the FITC labeling in the presence of ATP appeared covalent but labile.

Table II: Displacement or Removal of Labile FITC from the Na^+,K^+-ATPase[a]

FITC removal conditions		% fluorescence quenched by Ab	n
during labeling	postlabeling		
aminofluorescein	none	32 ± 8	2
		39 ± 2	3
	aminofluorescein	28 ± 2	2
	Seph 4B	13 ± 2	2
	anti-FITC/Seph 4B	0 ± 1	2
	dialysis	30	1

[a] Na^+,K^+-ATPase was labeled by 10 μM FITC, and unbound FITC was removed (see Materials and Methods). Several procedures were then used to remove the labile fraction of the enzyme-bound FITC. The labile probe not removed was assessed by the percent of fluorescence quenched by anti-FITC antibodies. FITC removal conditions were as follows: competition with a 3-fold excess of aminofluorescein (AF) during labeling; 30-min AF treatment after labeling, followed by separation of enzyme from the dissociated FITC; gel filtration chromatography on a Sepharose 4B (30 × 2.5 cm) column, or dialysis against 1 mg/mL BSA 72 h. FITC fluorescence quenching was determined following addition of 4 μg/mL antibody to 2 μg/mL labeled enzyme. n = number of experiments.

We then compared several procedures designed to eliminate this nonspecific labeling. After each treatment, the nonspecific fluorescence that remained was assayed as the percent of fluorescence quenched by the antibody. As shown in Table II, the presence of aminofluorescein, a nonfluorescent FITC analogue, had no effect on FITC labeling, while it did displace some FITC after the enzyme was labeled and washed. Extensive gel filtration on Sepharose 4B was more effective in removing FITC than our usual centrifugation and washing procedure, but 10–15% of the antibody-quenchable fraction still remained. Gel filtration in the presence of antibody was the most effective procedure to eliminate the conformation-insensitive probe.

FITC Fluorescence Decay Lifetime. In order to characterize more fully the two different FITC populations, frequency domain spectroscopic analysis was used to examine the fluorescence decay lifetime values of the enzyme-bound FITC. Lifetime data were obtained for the labeled enzyme (78 μg/mL) in Tris buffer (E_1 conformation) and then for each of three subsequent conditions: (1) 3 mM KCl ($E_1 \rightarrow E_2K^+$); (2) 100 μg/mL anti-FITC antibody (E_2K^+ plus Ab); then (3) 100 mM NaCl ($E_2K^+ \rightarrow E_1Na^+$ plus Ab); results are shown in Table III. The frequency domain data were fit by using nonlinear least-squares analysis software supplied by ISS Inc. (Urbana, IL). None of the phase and modulation data fit well to a model with a single-lifetime component. A significantly better fit was obtained when the data were fit to a model with one FITC fluorescence decay lifetime plus a fixed fast-lifetime component of 0.01 ns to account for light scattering from the visibily turbid enzyme solutions. When the data were fit to two-lifetime species plus a light scattering component, there was no improvement of the fit as assessed by the reduced χ^2_R.

Table III shows that the lifetime for FITC fluorescence decay was nearly identical under all conditions. Both the E_1 and E_2 enzyme conformations and both labeling populations gave lifetimes of 3.3 ± 0.2 ns versus 3.99 ± 0.05 ns for the free fluorophore.

Table III: Lifetime Analysis of FITC-Labeled Na^+,K^+-ATPase Preparations[a]

enzyme conformation	anti-FITC antibody	α_1	τ_1 (ns)	χ^2
One-Lifetime Fit: α = Fraction; τ = Lifetime[b]				
E_1Tris	−	1	2.83	31.7
E_2K^+	−	1	2.83	23.3
E_2K^+	+	1	2.24	25.1
E_1Na^+	+	1	2.87	35.35

enzyme conformation	anti-FITC antibody	α_1	τ_1 (ns)	α_2	τ_2 (ns)	χ^2
One-Lifetime plus Light-Scattering Fit[c]						
E_1Tris	−	0.80	3.30	0.20	0.01	2.18
E_2K^+	−	0.80	3.39	0.20	0.01	5.85
E_2K^+	+	0.67	3.50	0.32	0.01	4.34
E_1Na^+	+	0.90	3.06	0.10	0.01	2.29

[a] Decay lifetimes were measured as described under Materials and Methods with the FITC-labeled Na^+,K^+-ATPase in the E_1 and E_2 conformations with and without the antibody-accessible fluorescence quenched by anti-FITC antibodies. Conformation and Ab quenching fluorescence quenced by anti-FITC antibodies. Conformation and Ab quenching combinations were achieved by consecutive additions to a single enzyme sample (100 μg/mL) in Tris buffer (E_1Tris, −Ab); 2.4 mM KCl (E_2K^+, −Ab); 150 μg/mL anti-FITC Ab (E_2K^+, +Ab); plus 80 mM NaCl (E_1Na^+, +Ab). [b] τ is the nanosecond fluorescence decay lifetime calculated for a single fluorescence species; α is the fractional contribution of a particular τ, and χ^2_R is the goodness of fit parameter. [c] τ_2 was set to a fast, constant value (0.01 ns) to account for the light-scattering portion of the data, and analysis for a second lifetime (i.e., two fluorescence components plus a light-scattering component) yielded no better fit.

Table IV: Determination of Relative Quantum Yield and Absorbance Maxima for FITC and Na^+,K^+-ATPase-Bound FITC[a]

sample	relative quantum yield	λ_{max}
FITC	1.0 ± 0.06	492
FITC–Na^+,K^+-ATPase		
E_1Na^+ conformation	0.5 ● 0.1	497
E_2K^+ conformation	0.33 ± 0.06	497
FITC–Na^+,K^+-ATPase (trypsin digested)	0.86 ± 0.02	494
FITC–Na^+,K^+-ATPase (+ATP)	0.93 ± 0.08	494

[a] The relative quantum yield (fluorescence:absorbance ratio) was taken as the fluorescence intensity divided by the absorbance for each sample and normalized to the value for free FITC. Absorbance values and the wavelength of maximum absorption (λ_{max}) were taken from spectra of 100 and 200 μg/mL labeled enzyme with the base-line turbidity subtracted. Fluorescence was measured at 520-nm wavelength with excitation at 495 nm for enzyme samples of 5 μg/mL protein or 2 nM FITC. The E_1Na^+ and E_2K^+ conformations were induced by 10 mM NaCl and 10 mM KCl, respectively. Trypsin digestion was accomplished with 1:10 trypsin/enzyme (w/w) 27 °C, 4 h, pH 8.0; ATP-protected samples were labeled in the presence of 5 mM Na_2-ATP.

Since the fluorescence lifetimes but not the intensities of the enzyme-bound probe in the E_1 and E_2 conformations were found to be constant, mechanisms other than collisional quenching to explain the ligand-induced fluorescence changes were investigated. Static quenching of fluorescence (i.e., a lifetime-independent event) was detected by determining the fluorescence:absorbance ratios or relative quantum yields of the probe populations. By normalization of fluorescence to absorbance, the effects of potential differences in the extinction coefficients were eliminated. Table IV shows that the quantum yield of FITC at the active or ATP-blockade site was about 50% that of free FITC. Additional quenching then occurred upon the addition of K^+ to effect the E_1Na^+ to E_2K^+ conformation transition. This static quenching appears to account quantitatively for some of the commonly observed ligand-induced fluorescence intensity changes. Control samples, enzyme FITC-labeled with ATP present, showed quantum yields similar to that of free FITC.

Solvent Effects on the Active-Site-Linked FITC. Solvent effects upon the spectral properties of the antibody-inaccessible FITC population were used to characterize this site further. Table IV shows that the absorption spectra of FITC bound at the conformation-sensitive site are red-shifted 5 nm relative to that of free FITC and 3 nm relative to that of the population labeled in the presence of ATP. Subjecting the enzyme to trypsin digestion then reversed this change with the quantum yield and λ_{max} of FITC shifting back to those of the antibody-accessible population. The environment at the conformation-sensitive site appears to be similar to that observed for FITC in ethanol relative to water (Martin, 1975) and correlates with the probe residing in a hydrophobic pocket of the protein. However, the E_1Na^+ to E_2K^+ conformational change had no additional effect on the absorbance spectrum of the active-site-linked FITC.

To determine if the protein's ionic environment about the probe was responsible for the ligand-induced alterations in the fluorescence intensity of the active-site-bound probe, its fluorescence was measured as a function of pH. At pH values between 3 and 9.2, both conformations had similar fluorescence intensity increase curves with pK_a's of about 6.1 which, within experimental error, were identical with that seen for free fluorescein. Furthermore, no other fluorescence intensity inflections were observed, indicating no shift in the probe's ionization profile and an absence of titratable ionic amino acid residues directly interacting with FITC.

However, changes in the access of bulk solvent affect the exchange of protons with probe, and this may alter its fluorescence intensity. Voss et al. (1980) have shown that D_2O can enhance the fluorescence of antibody-bound FITC. In these studies, we found that D_2O (90%) did not affect the fluorescence of free FITC but that the fluorescence of active-site-bound FITC increased 26% for the E_1Na^+ conformation and 40% in the E_2K^+ conformation. This indicated conformation-dependent differences in the probe's environment resulting from specific interactions between FITC and nearby amino acids.

Iodide Quenching Effects on FITC Fluorescence. In addition to the D_2O enhancement effects, collisional quenching of FITC fluorescence can be highly sensitive to the solvent accessibility of the fluorophore because it requires the close approach of the quencher and fluorophore. However, the lifetime of fluorescence decay (3–4 ns) must be long enough for the diffusional approach of quencher over a relatively large distance (≥10 nm). Consequently, fluorophores buried within proteins or in crevice sites will have lower quenching constants than highly exposed molecules because the diffusional approach of the quencher is sterically blocked. In these studies, we used I^- (choline iodide) as a negatively charged collisional quencher (Eftink & Ghiron, 1981). Three protein preparations were used in order to compare the two FITC labeling sites on Na^+,K^+-ATPase: (1) enzyme fully labeled in the absence of ATP; (2) the fully labeled enzyme to which anti-FITC antibodies were bound; (3) protein labeled in the presence of ATP.

Table V shows the quenching constants (K_q) for the three protein samples as derived from the Stern–Volmer analysis of fluorescence quenching data obtained with choline iodide. For the antibody-accessible site (sample 3), K_q = 6.2 ± 0.2 M^{-1}, and quenching was insensitive to the E_1 to E_2 conformational change. In contrast, the fully labeled enzyme had a quenching constant of K_q = 4.7 M^{-1} while K_q for the conformation-sensitive FITC population was 3.4 M^{-1} for E_1Na^+

Table V: Iodide Quenching Constants (K_q) for FITC-Labeled Na^+,K^+-ATPase[a]

FITC labeling conditions	sites present: antibody	sites present: conformation	K_q values for enzyme conformation: E_1Na^+	K_q values for enzyme conformation: E_2K^+
+ATP	+	-	6.2 ± 0.2	6.3 ± 0.1
-ATP	+	+	4.7 ± 0.2	4.0 ± 0.3
-ATP + anti-FITC Ab	-	+	3.4 ± 0.1	1.8 ± 0.2
Values Calculated from -ATP Conditions				
-ATP	+	-	6.6 ± 0.5	6.0 ± 1.0
-ATP	-	+	3.7 ± 0.4	1.7 ± 0.26

[a] The FITC-labeled Na^+,K^+-ATPase contained two FITC populations. Labeling with 10 μM FITC in the presence of 5 mM ATP (+ATP) selectively blocked the conformation-sensitive site. Labeling in the absence of ATP (-ATP) labeled both sites, but when anti-FITC Ab was added (-ATP + anti-FITC Ab), only the conformation-sensitive site was observed. These samples were titrated with choline iodide (0–100 mM), and the resultant fluorescence quenching curves were analyzed by the Stern–Volmer equation. Although two quenching constants contributed to the -ATP sample, they were calculated individually by using eq 2 under Materials and Methods for comparison to the values obtained under -ATP + anti-FITC Ab and +ATP conditions. All quenching curves were linear ($R > 0.99$) and consisted of seven points, and the derived values of K_q were the average of triplicate determinations.

and was sensitive to enzyme conformational changes (E_2K^+ = 1.8 M^{-1}). The K_q values show that the conformation-sensitive site is quenched with only about 50% of the efficiency of the antibody-accessible site. This suggests that this site is significantly shielded from solvent iodide, and even further shielded in the E_2 conformation.

In doing these studies, it was necessary to show that the bound antibody did not alter the approach of solvent iodide ions causing artificially low quenching constants. We therefore also calculated the quenching constants of the two populations of sites in the absence of the antibody. This was possible because the quenching constant for the fully labeled enzyme (K_{q12}) is a linear combination of the quenching constants for the two contributing sites (see eq 2 under Materials and Methods):

$$K_{q12} = f_1K_{q1} + f_2K_{q2} \quad (2)$$

where K_{q1} and K_{q2} represent the values for the conformation-sensitive and antibody-accessible sites, respectively. The fractional fluorescence intensity of both sites is then f_1 and f_2 (see Materials and Methods). When the measured values of f_1, f_2, K_{q2}, and K_{q12} (from Table V) were used, the calculated K_{q1} was 3.7 M^{-1} in the E_1 conformation and 1.7 M^{-1} in the E_2 conformation. These values agree well with the data obtained in the presence of antibody. Similarly, the value of K_{q2} as calculated from the measured values of f_1, f_2, K_{q1}, and K_{q12} was nearly identical with the K_{q2} (6.0) measured with antibody present. Clearly, the population of antibody-accessible FITC sites is at a highly exposed region that is distinct from the conformation-sensitive site, and the anti-FITC antibody quenches the fluorescence at these sites without affecting the quenching properties of the latter.

Since local ionic charges near the FITC binding sites may affect the approach of a negatively charged iodide, quenching experiments with uncharged molecules were also done, but neither acrylamide nor *N*-methylnicotinamide appreciably quenched enzyme-bound FITC. The positively charged quencher Cs^+ was also tried, but it altered the enzyme conformation, and little or no additional quenching was observed.

Anisotropy Determinations. The extent to which the fluorescence emission from a fluorophore excited with polarized light is also polarized can reveal the rotational freedom of the probe. In order to assess specifically the rotational freedom of the fluorescein molecules in the conformationally sensitive labeling site, polarization measurements were made as the labeled protein was titrated with anti-FITC antibodies. Antibody binding, as monitored by the decrease in FITC fluorescence intensity, was accompanied by an increase in probe anisotropy (r) as shown in Figure 4. Using the data from Figure 4 to determine the proportion of the fluorescence intensity contributed by each probe population, we calculated the r of FITC at the antibody-accessible site as 0.10 ± 0.05, which is substantially higher than FITC free in solution (0.01). The anisotropy for the probe at the conformation-sensitive site was 0.336 ± 0.001 and after SDS denaturation of the enzyme 0.276. The r value of 0.336 approaches that expected in the absence of rotational motion (r_0 = 0.390; see Materials and Methods), indicating that very little rotation is allowed. The average angle of probe rotation or displacement (θ) occurring during the lifetime of fluorescence emission (τ = 3.1 ns) was then calculated by using the ratio of the two anisotropy values (r/r_0) and the following form of the Perrin equation, $r/r_0 = (3\cos^2\theta - 1)/2$, and found to be 17°. The steady-state anisotropy, however, is dependent both on the rate of probe displacement and on the limiting angle over which it can rotate during the lifetime of the emission. The contributions of these effects were modeled by an expression of the Perrin equation derived for hindered rotators (Lakowicz et al., 1979):

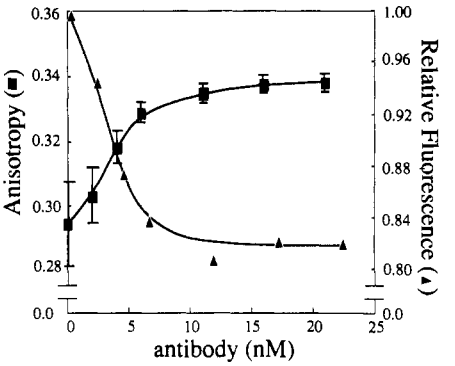

FIGURE 4: Effect of anti-FITC antibodies on the anisotropy and relative fluorescence intensity of FITC–Na^+,K^+-ATPase. Thirty-three micrograms of labeled enzyme was titrated with increasing concentrations of antibody. The decrease in fluorescence intensity is shown in the closed triangles' curve (▲) while the increase in anisotropy is shown by the closed squares' curve (■). The anisotropy values are the averages of three determinations, while a representative curve of the fluorescence intensity is shown.

$$r = r_\infty + (r_0 - r)/(\tau/\phi) \quad (9)$$

Here ϕ is the rotational correlation time of the probe, and τ is the emission lifetime. Also, r_∞ is the limiting anisotropy at long times and is related to the average of displacement (θ') of fluorescein in the FITC binding site:

$$r_\infty/r_0 = (3\cos^2\theta' - 1)/2 \quad (10)$$

In order to determine the values for r_∞ and ϕ, the anisotropy (r) of FITC at the conformation-sensitive site was measured at different quencher (I^-) concentrations. Then since the degree of I^- quenching is directly related to a decrease in the fluorescence decay lifetime, using eq 9, the data were plotted as r vs $r_0 - r/\tau$ (not shown). The slope of the best-fit line gives ϕ, while r_∞ is taken from the y intercept and used in eq 10 to calculate θ', the average limiting angle of displacement. The data gave an average value for ϕ of 0.1 ns, but the large standard deviation in this value (±0.2–±0.4 ns) revealed that

Table VI: Analysis of the Effect of Enzyme Conformation on the Hindered Rotation of Na^+,K^+-ATPase-Bound FITC[a]

conformation	ϕ (ns)	r_∞	θ' (deg)	N
E_1Na^+	0.1 ± 0.2	0.340 ± 0.005	17 ± 2	18
E_2K^+	0.1 ± 0.4	0.341 ± 0.008	17 ● 2	18

[a] The anisotropy (r) of the FITC-labeled enzyme was measured in the presence of 10 mM NaCl (E_1Na^+) or 10 mM KCl (E_2K^+) over a range of fluorescence decay lifetimes ($\tau \approx 3.4$–1.7 ns). Lifetimes were modulated by quenching with choline iodide (see Materials and Methods). These data were plotted according to the Perrin equation modified for hindered rotation: $r = r_\infty + (r_0 - r)\phi/\tau$. The intercept from the plot of r vs $(r_0 - r)/\tau$ was used to estimate the lifetime-independent limiting anisotropy (r_∞), and the rotational correlation time of the probe (ϕ) was estimated from the slope. Although the value from ϕ was 0.1 ns, the error in ϕ makes it essentially indistinguishable from zero with 0.5 ns as its upper limit. Finally, r_∞ was used to calculate the average angle of probe displacement (θ') (Lakowicz et al., 1979): $r_\infty/r_0 = (3\cos^2\theta' - 1)/2$. N is the number of data points used to calculate ϕ and r_∞.

this value was within a range from 0 ns (or slope = 0) to 0.5 ns (see Table VI). This is much faster than the FITC fluorescence lifetime and indicates that the steady-state anisotropy depends mainly on r_∞. Table VI also shows r_∞ calculated from the same data. The average limiting angle of displacement was then found to be 17 ± 2°, suggesting that FITC is in a highly restricted environment. Table VI also shows that the $E_1Na^+ \rightleftarrows E_2K^+$ conformational change did not alter FITC anisotropy nor have any effect on its motional restriction.

Fluorescence Energy-Transfer Measurements. Previously, the distance between FITC and the cardiac glycoside binding site was measured by using the ability of FITC to quench the fluorescence of bound anthroylouabain by energy transfer (Carilli et al., 1982). We repeated these measurements in order to find the contribution of each FITC population to this quenching. In these experiments, the fluorescence emission spectra of anthroylouabain (AO) bound to the FITC-labeled enzyme and bound to the unlabeled enzyme were compared. The presence of FITC was found to reduce AO fluorescence by only 3%, and this reduction was unaffected by the binding of anti-FITC antibody. Therefore, the antibody-accessible fraction of the enzyme-linked FITC fluorescence did not contribute to the energy-transfer measurement of the distance between AO and FITC.

Discussion

Substantial evidence indicates that structural rearrangements or conformational changes occur in the Na^+,K^+-ATPase during the catalytic process. These changes affect the enzyme affinity for its ligands, cause a reorientation of bound ATP necessary for its hydrolysis, and accomplish occlusion and translocation of Na^+ and K^+ across the cell membrane. In these experiments, we investigated the spectral properties of the enzyme-linked fluorescent probe FITC in order to detect changes in the environment about this probe which may account for the changes in its fluorescence intensity upon the $E_1 \rightleftarrows E_2$ conformational transitions. Because it is likely that the xanthone moiety of FITC resides at least partially in the adenine binding site, these studies should give a better understanding of the complexities of ATP binding and the reaction mechanism of the enzyme.

In these studies, we have found that FITC-directed antibodies bind to and quench the fluorescence of a population of FITC molecules that appear to be covalently, yet reversibly, bound to the enzyme. Consistent with other studies, this population of FITC is removed from the denatured protein by gel electrophoresis (Figure 3). While its lability had kept it undetected in earlier sequencing studies, in spectroscopic studies its presence is significant. Amler et al. (1989) have, for example, reported FITC labeling of Na^+,K^+-ATPase in the presence of ATP that had a high degree of anisotropy (0.12), indicating it was not simply free, unbound probe. In addition, recent sequence studies by Xu (1989) report that at the labeling stoichiometries of FITC/enzyme of 1:1 and 1.7:1 that only 57% of the enzyme-bound FITC fluorescence was recovered bound to tryptic fragments and that 30% of the peptide labeling can occur in the presence of 3 mM ATP. Further, we have found that it is the antibody-inaccessible fraction of FITC that is sensitive to enzyme conformational changes (Figure 1) and whose labeling is blocked by ATP (Figure 3). This suggests that it is the FITC molecules binding at Lys-501 that serve as the reporter group probe and that they are highly sequestered in the protein.

Consistent with our results, Mata et al. (1989) have reported that the fluorescence of FITC bound to the sarcoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase (0.3 mol of FITC/mol) was not quenched by the addition of FITC-directed antibodies, while Swoboda and Hasselbach (1985), in making a thorough study of the reaction products of FITC with the Ca^{2+},Mg^{2+}-ATPase found a labile component that had the absorption characteristics of a sulfhydryl derivative. These reports are supportive of our data, but while the two enzymes are similar, they do not have identical sequences at their respective FITC-reactive site(s). Also the fluorescence intensity changes (4–12%) undergone by Ca^{2+}-ATPase-bound FITC (Highsmith, 1986) and its polarization values (r = 0.056; Squier et al., 1987) are considerably lower than those of the Na^+,K^+-ATPase.

In the context of these studies, the anti-FITC antibodies have proven extremely useful; they leave the remaining inaccessible probe's fluorescence intensity, solvent accessibility, anisotropy, and lifetime unchanged, and have no effect on the enzyme's ability to undergo its conformational changes.

We have therefore been able to demonstrate that the environment of the FITC-labeled enzyme at the putative ATP binding site is apparently hydrophobic. The red shift in the FITC absorbance spectrum suggests a polar environment similar to that of ethanol or methanol (Martin, 1975) which is not further altered by enzyme conformation changes (see Table IV). In addition, we have demonstrated specific probe and protein interactions at this binding site region. For example, the deuterium isotope effect is specific for FITC at this site. Also, FITC fluorescence is quenched by reacting at this site and then reduced further by the $E_1 \rightarrow E_2$ conformational transition (Table IV). The initial quenching of FITC appears to be about equally caused by dynamic (lifetime dependent, see text) and static processes, while the second occurs by a strictly static mechanism that cannot be accounted for by general solvent effects (Table III). Finally, FITC fluorescence is probably not affected by a specific charged amino acid residue in this site because pH titrations showed no shift in the pK_a of ionization for bound FITC or for either the E_1Na^+ or the E_2K^+ conformations.

A structural basis for these observations is suggested by model studies investigating the quenching of fluorescein by amino acids. Tyrosine and tryptophan were shown to be the most efficient quenchers (Watt & Voss, 1977). If either of these two residues is responsible for the quenching, then we would predict that it should reside on the intracellular side of the membrane between the fourth and fifth transmembrane sequences and not be flanked by amino acids with titratable charged groups. Further, since the $E_1 \rightleftarrows E_2$ induced fluorescence changes for the Ca^{2+},Mg^{2+}-ATPase (Pick &

Karlish, 1980) are smaller and opposite to that of Na^+,K^+-ATPase, the Tyr or Trp residue-containing sequences should probably be in a nonconserved region. These considerations give three amino acids from the sheep kidney enzyme: Trp-385, Trp-411, and Tyr-535. Each of these positions is conserved among different species of Na^+,K^+-ATPase and in the gastric H^+,K^+-ATPase which has similar FITC-monitored $E_1 \rightleftarrows E_2$ transitions.

In our work, the conformation-sensitive labeling population does appear to be homogeneous. Its labeling stoichiometry is less than one per $\alpha\beta$ promoter, only one fluorescence decay lifetime or polarization component was observed, and its relative $E_1 \rightleftarrows E_2$ fluorescence intensity change was constant at all labeling concentrations used. Our data, however, cannot rule out the possibility that the labeling is distributed between several reactive residues but with the xanthone moiety residing within the same fluorescein binding site as suggested by Xu (1989).

The probe at this site is, though, divided into quenched and unquenched populations. These were distinguished by the observation of partial static quenching. Since this process is faster than fluorescence decay, it should quench with 100% efficiency (Lakowicz, 1983), and the remaining fluorescence is therefore from a different population of probe. Conformational changes in the enzyme subsequently cause reversible changes in the relative distribution between the two probe populations, with the quenched conformation more highly favored in the E_2 than E_1 conformation.

Altogether our data support the view that this labeling site is in a protected pocket or cleft that has limited access to the bulk solvent and which should include some portion of the ATP binding site. Additional solvent restriction occurs upon the $E_1Na^+ \rightarrow E_2K^+$ transition, but there are no additional decreases in either the probe's highly restricted rotational flexibility or the polarity of the probe's hydrophobic environment. FITC in this site interacts with the immediate residues of α in a complex way that more closely resembles that of the antibody–hapten interactions of FITC, which are also accompanied by quenching, than that of FITC coupled to a protein like albumin (Watt & Voss, 1978). The Na^+,K^+-ATPase may have a nucleotide pocket, similar to that found in the crystal structure of adenylate kinase or other ATPases that bind Mg^{2+}ATP, which undergoes dramatic ligand-dependent affinity changes through the restriction of access to the site (Fry et al., 1985; Jorgensen & Anderson, 1988; Moczydlowski & Fortes, 1981; Smith et al., 1980).

While these studies suggest that FITC may remain in a relatively fixed position, our previous studies have suggested that the Mg^{2+}ATP complex binds initially at a high-affinity site (E_1 conditions) and then that a repositioning of ATP must take place prior to enzyme phosphorylation and ATP hydrolysis (Ball, 1986). In addition, we have also found that the HLLVMKGAPER sequence region of α which includes the Lys-501 is sensitive to ligand-induced changes in enzyme conformation (Ball & Friedman, 1987). It may be that movement of this region in response to ligands relocates the attached FITC and thereby promotes the changes in fluorescence intensity. The lack of any $E_1 \rightleftarrows E_2$ changes in probe anisotropy would indicate that the probe is not, however, moving from an exposed to a buried location in the nucleotide binding site. An alteration within the nucleotide binding site could also be responsible, though, for the changes in the static quenching of FITC.

Certainly it is a specific combination of circumstances including both its site of labeling and the physical properties of the probe that combine to make the antibody-inaccessible population of FITC so uniquely sensitive to enzyme conformation in comparison to the many other fluorescent probes that have been used with Na^+,K^+-ATPase. Hopefully, the use of various fluorescein derivatives, different isoforms of the enzyme, and site-specific mutational varients of the Na^+,K^+-ATPase will yield substantial additional information about which regions of α interact with FITC.

An important implication of our work is that the labeling site(s) of the antibody-accessible probe is (are) unknown and any energy transfer studies done using FITC as one of the pair of fluorophores could be very sensitive to small levels of exposed, less rotationally restricted, FITC which might reside closer to the other molecule than the Lys-501-linked FITC population. Our initial studies done using anthroylouabain and FITC have shown that the extent of energy transfer between these two probes which are on opposite sides of the cell membrane is at the very limits of the sensitivity of this technique. Only about a 3% decrease in anthroylouabain fluorescence was observed, and the presence of anti-FITC antibodies did not change this significantly. Therefore, the results of Carilli et al. (1982) and Jesaitis and Fortes (1980), who have previously reported energy transfer values at this level, would not seem to be affected by the observed heterogeneity in the FITC labeling. However, for probes which both lie on the intracellular side of the enzyme, we would expect that there could be serious errors in the analysis of fluorescence energy-transfer data if both FITC populations are not taken into account.

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