Characterization of the Properties of Ethenoadenosine Nucleotides Bound or Trapped at the Active Site of Myosin Subfragment 1†

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ABSTRACT: The fluorescent nucleotide analogue of ADP, 1,N^6-ethenoadenosine diphosphate (ϵADP), has been used to probe the active site of myosin subfragment 1 (SF_1). The Mg complex of ADP was shown to be trapped stoichiometrically at the active site by a variety of thiol cross-linking agents having sulfur to sulfur spanning lengths of 2–14 Å. Previous studies [Wells, J. A., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966–4970] had suggested ADP was trapped by direct closure of a postulated active site cleft by cross-linking two activity critical thiols, SH_1 and SH_2. This model was tested by measuring the polarization of trapped and reversibly bound ϵADP, the off-rate of trapped ϵADP, and the solute quencher accessibility of trapped ϵADP on SF_1 modified with thiol cross-linking agents of different spanning lengths. The lack of correlation of all of these properties with the length of the cross-linking span suggests that trapping occurs by indirect stabilization of a conformation favoring bound nucleotides rather than by sterically preventing the release of nucleotide. Measurement of the fluorescent properties of ϵADP bound to SF_1 vs. ϵADP free gave a 20% increase in emission intensity, a 7-nm blue shift in the emission maximum, and a 70% increase in the absorbance at the excitation wavelength (330 nm). Trapping of ϵADP by the thiol cross-linking agent *p*-phenylenedimaleimide gave a further 24% increase in emission intensity. This change was shown to be the result of an increase in absorbance of trapped ϵADP at 330 nm rather than an increase in the quantum yield. The Stern–Volmer quenching constants for I^- and acrylamide were determined for ϵADP free in solution, bound reversibly, or trapped on SF_1 by cross-linking agents. These constants decreased in both systems by a factor of 3 for I^- and 10 for acrylamide, respectively, indicating that the purine ring was buried and removed from the bulk solvent. Preliminary lifetime studies with thiol chromophoric cross-linking agents trapping ϵADP indicate Förster energy transfer is occurring and suggest it should be possible to measure the distance from cross-linked thiols to the active site.

A widely used approach in the study of nucleotide-binding proteins has been the use of fluorescent nucleotide analogues (Stryer, 1978). Of these, the 1,N^6-ethenoadenosine derivatives such as ϵATP[1] (Secrist et al., 1972) have proven by far to be the most useful [for review, see Leonard (1984)]. Among the desirable properties of these analogues are (i) a long fluorescence lifetime, (ii) a high quantum yield, (iii) a high intrinsic polarization, (iv) and excitation and emission spectrum removed from those of proteins and nucleic acids, (v) a high degree of structural similarity to the parent adenine containing compounds, (vi) a marked dependence of lifetimes on the dielectric constant and pH of the medium, and finally (vii) a high degree of photostability (Secrist et al., 1972; Penzer, 1973; Spencer et al., 1974).

ϵATP and ϵADP have been widely used in studies of the contractile protein, myosin. Initial studies showed ϵATP was an effective substrate (McCubbin et al., 1973), that it bound to a single site on each head of myosin (Willick et al., 1973), and that it supported contraction of muscle fibers (Mowery, 1973). Onishi et al. (1973) measured some of the spectral characteristics of ϵATP bound to myosin, further examined the kinetic properties of ϵADP as a substrate, and found indications of Förster energy transfer between enzyme tryptophan(s) and bound ϵATP. Goody and co-workers (Goody et al., 1981; Konrad & Goody, 1982) have used the 20% decrease in tryptophan fluorescence that occurs with addition of ϵADP (possibly Förster energy transfer) to do competitive binding studies of other nucleotides to SF_1. The kinetics and mechanism of binding of ϵADP and ϵATP to SF_1 and HMM were extensively examined by Garland & Cheung (1976, 1979). Subsequently, Burke (1980) showed the addition of ϵADP stimulated the rate of alkylation of SH_2. It was, however, less effective than ADP, indicating that the conformation about SH_2 differed slightly in the presence of the two nucleotides. Useful structural information about cross-bridge orientation in glycerinated muscle fibers has also been obtained by polarization studies of etheno nucleotides bound to myosin (Yanagida, 1981). These studies showed that the distribution of cross-bridge orientations varied both with the physiological state of the fiber and with the nature of the nucleotide added. Most recently Ando et al. (1982) and Rosenfeld & Taylor (1983) have used the selectivity of acrylamide quenching to monitor specifically the fluorescence of ϵADP bound at the active site of myosin. This new approach promises to be a useful probe of the various states present during the catalytic cycle of the myosin and actomyosin ATPase.

The observation that a variety of nucleotides and nucleotide analogues could be stably trapped at the active site of myosin by bifunctional thiol cross-linking agents [for review, see Wells & Yount (1982)], suggested that appropriate etheno nucleotides would also be trapped. This approach would facilitate the complete characterization of bound nucleotide since all

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[1] Abbreviations: ϵATP, 1,N^6-ethenoadenosine triphosphate; ϵADP, 1,N^6-ethenoadenosine diphosphate; ϵAMP-PNP, 1,N^6-ethenoadenyl-5′-yl imidodiphosphate; NDM, naphthylene-1,5-dimaleimide; pPDM, *N,N′-p*-phenylenedimaleimide; oPDM, *N,N′-o*-phenylenedimaleimide; F_2DPS, 4,4′-difluoro-3,3′-dinitrodiphenyl sulfone; F_2DNB, 1,5-difluoro-2,4-dinitrobenzene; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); phen, 1,10-phenanthroline; SF_1, myosin subfragment 1; ATPase, adenosinetriphosphatase; Tris, tris(hydroxymethyl)aminomethane.

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free nucleotide could be removed by conventional gel filtration. In addition, the etheno nucleotides could act as probes to elucidate possible differences between reversibly bound and trapped nucleotides. Moreover, the fluorescent properties of ϵADP trapped by a variety of different sized thiol cross-linking agents should allow testing of the simple JAW model proposed to explain trapping (Wells & Yount, 1979). This latter model postulated that cross-linking SH_1 and SH_2 directly closed a nucleotide binding cleft. This paper evaluates the JAW model and presents a detailed description of the fluorescent properties of ϵADP reversibly bound or trapped on SF_1.

Materials and Methods

Materials

The sodium salts of ϵADP and ϵATP were from P-L Biochemicals. ϵATP was prepared as its sodium salt by the method of Secrist et al. (1972). ϵAMP-PNP was prepared as its sodium salt by coupling ϵAMP with imidodiphosphate by the procedure of Hoard & Ott (1965). This synthetic approach was required because the P–N bonds of AMP-PNP were not stable under the pH 4 conditions of the etheno derivatization. ADP and NDM were from Molecular Probes (Junction City, OR). The purity of the etheno nucleotides was confirmed by reverse-phase high-performance liquid chromatography (Mahoney & Yount, 1984). All etheno nucleotide solutions used were >95% pure. Less than 5% impurity was found in up to 5-day-old Tris–KCl, pH 8, solutions of the etheno nucleotides stored on ice. oPDM, pPDM, and 1,10-phenanthroline (phen) were from Aldrich. The cross-linking reagents F_2DPS, F_2DNB, and DTNB were from Pierce. $CoCl_2 \cdot 6H_2O$, KI and $N_2S_2O_3 \cdot 5H_2O$ were Mallinckrodt analytic reagent grade. Bis(phenanthroline)(carbonato)cobalt(III) was prepared according to Ablov & Palade (1961) as previously described (Wells et al., 1979).

Rabbit skeletal myosin was prepared according to Wagner & Yount (1975). Chymotryptic subfragment 1 (SF_1) was prepared according to Weeds & Taylor (1975) and was assumed to have a molecular weight of 115000 (Weeds & Pope, 1977).

Methods

Analytical Procedures. NH_4^+-EDTA-ATPase and Ca^{2+}-ATPase activities were measured as described (Wells et al., 1979). Magnesium was determined by atomic absorption (Perkin-Elmer 360) as described (Wells et al., 1980a). The protein concentration of chemical modified SF_1 solutions was determined by the Coomassie blue dye binding method (Bradford, 1976). Unmodified SF_1 was used as a protein standard with $E_{280}^{1\%} = 7.5$ cm^{-1} (Wagner & Weeds, 1977).

Enzyme Inactivations. SF_1 (17 μM) in 0.1 M KCl and 50 mM Tris, pH 8.0 (KCl–Tris buffer), at 0 °C was incubated in the presence of 2 mM $MgCl_2$ and 0.2 mM ATP or etheno nucleotide. Inactivation of SF_1 by cobalt chelation employed a 100-fold excess of $[Co^{III}(phen)_2CO_3]^+$ and a 10-fold excess of Co^{II}(phen) (Wells et al., 1979). pPDM, F_2DPS, and F_2DNB inactivations used a 1.3-fold excess of reagent. NDM, oPDM, and DTNB were used in 1.4-, 1.5-, and 2-fold excess over SF_1, respectively. Inactivations using F_2DNB and DTNB required approximately 24 h to go to completion. All other inactivation systems were stopped after a 25-min reaction time.

Enzyme derivatives were purified from excess and unreacted modifying reagents, nontrapped nucleotide, and divalent cation by precipitation with 2.5 volumes of saturated $(NH_4)_2SO_4$ (pH 8.0) containing 20 mM EDTA. Protein pellets obtained after centrifugation were dissolved in 0.5 mL of KCl–Tris buffer, applied to a 1 × 10 cm Sephadex G-25 column (Pharmacia), and eluted with KCl–Tris buffer at 4 °C.

Steady-State Fluorescence Measurements. Steady-state fluorescence measurements were made with an SLM 4800 spectrofluorometer equipped with Glan Thompson prism polarizers and a thermostated cuvette turret. An excitation wavelength of 330 nm and excitation and emission slit widths of 4 nm were generally used. All solutions used in making fluorescence measurements were filtered on Millex-PF 0.8-μm filter units to minimize light scattering contributions to the emission intensity.

Corrected fluorescence emission spectra were measured by using 4-nm excitation and emission slit widths. Correction factors were obtained by calibrating the emission monochromator and photomultipler with a tungsten light source of known spectral distribution and with the emission polarizer in the vertical position. To eliminate the polarization bias of emission intensity measurements and to permit use of the correction factors, emission spectra were measured in two ways: (i) the excitation and emission polarizers were set at 55° and vertical, respectively (Spencer & Weber, 1970), and (ii) I_{VV}, I_{VH}, I_{HV}, and I_{HH} were measured, where the first term in the subscript denotes the excitation polarizer position and the second term refers to the emission polarizer position (V and H represent vertical and horizontal settings, respectively). The intensity is given by Curie's law of symmetry, $I_{total} = I_{\parallel} + 2I_{\perp} = I_{VV} + 2(I_{HV}/I_{HH})I_{VH}$ (Azumi & McGlynn, 1962; Cehelnik et al., 1975). Water Raman scattering intensity and SF_1 scattering intensity were subtracted from the emission spectrum of the $SF_1 \cdot \epsilon$ADP complex in all cases.

Single wavelength polarization measurements were made with Glan Thompson prism polarizers and were corrected for selective transmission characteristics of the emission monochromator (Azumi & McGlynn, 1962). A further correction was obtained by subtracting out polarization emission intensity contributions from tryptophanyl emission and light scattering of an SF_1 blank.

Aliquots of the two fluorescence quenching agents used, potassium iodide and recrystallized acrylamide, were taken from one M stock solutions. The iodide solution contained 10^{-4} M thiosulfate to prevent oxidation of I^- to I_3^- (Lehrer, 1971). Complete mixing of the quenching agent was assured by continuous magnetic stirring of the solution in the cuvette. The fluorescence quenching was measured with excitation and emission wavelengths of 320 and 410 nm, respectively. No change in the general shape or maximum of the emission spectrum occurred upon addition of the quenching agents in the concentration range used. Ionic strength effects on the enzyme fluorophore complex, measured by substituting KCl for KI solutions, were negligible.

Fluorescence Lifetime Measurements. Fluorescence lifetimes were measured by using an excitation wavelength of 320 nm and a Schott KV 408 cutoff filter to select for emission. To obtain emitted light that was independent of the anisotropic rotations of the $SF^1 \cdot \epsilon$ADP complex, the emission was observed without an emission polarizer and with the excitation polarizer set at 35° (Spencer & Weber, 1970). The instrument measured the fluorescence lifetime of a sample from the phase shift and demodulation of sample-emitted light with respect to a standard (e.g., a glycogen solution), having a scattered light intensity comparable to the fluorescence intensity of the sample. The modulation frequency used for most measurements was 6 MHz, which minimized any color–delay error (Jameson & Weber, 1981). An HP 9825A desktop computer interfaced to the fluorometer was used to calculate the fluorescence lifetimes. As a test for single exponential fluorescence decay,

Table I: Stoichiometries of Mg^{2+} and ϵADP Trapped on SF_1 by Different Cross-Linking Reagents[a]

cross-linking reagent	ATPase (%) NH_4^+-EDTA	ATPase (%) Ca^{2+}	Mg^{2+}/SF_1	ϵADP/SF_1[b]
control	97	90	0.03	0.00 (0.00)
Co^{II}phen	1	0	0.77	0.73 (0.83)
pPDM	22	46	0.55	0.70 (0.75)
oPDM	34	40	0.43	0.46 (0.46)
NDM	28	64	0.45	0.59 (0.61)
F_2DPS	11	31	0.48	0.62 (0.57)
DTNB	12	27	0.55	0.56 (0.53)

[a] Conditions: see Enzyme Inactivations under Materials and Methods. The initial NH_4^+-EDTA and Ca-ATPase values were 11.0 and 1.1 μmol min^{-1} (mg of $SF_1)^{-1}$, respectively. [b] ϵADP was determined by its fluorescence intensity after precipitation of SF_1 by addition of 0.1 volume of concentrated $HClO_4$ to the SF_1 solution. The values in parentheses were the same measurement except that protein was denatured by heating SF_1 solutions (30 min at 95 °C in a steam bath) and removed by centrifugation.

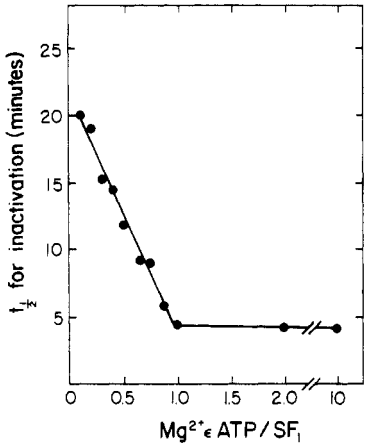

FIGURE 1: Effect of increasing ϵATP concentration on apparent half-time of Co^{II}/Co^{III}phen inactivation of SF_1. SF_1 (17 μM) was inactivated with a 5-fold excess of Co^{II}phen and a 50-fold excess of $[Co^{III}(phen)_2CO_3]^+$ at 0 °C in 20 mM $MgCl_2$ and KCl–Tris buffer, pH 8.0. The $t_{1/2}$ for inactivation was determined from a plot of the log percent residual NH_4^+-EDTA-ATPase activity vs. time.

the lifetimes determined by phase lag (τ_p) and demodulation (τ_m) were compared. Single exponential decay is indicated when τ_p is equal to τ_m (Spencer & Weber, 1969).

Results

A variety of thiol cross-linking reagents were effective in inactivating both the NH_4^+-EDTA-ATPase and Ca^{2+}-ATPase activities in the presence of excess ϵATP (Table I). Concurrent with the inactivation both Mg^{2+} and ϵATP were trapped, the latter probably in the diphosphate form, as was observed previously in studies using ATP (Wells & Yount, 1979). With complete inactivation the amount of magnesium and nucleotide trapped is nearly stoichiometric, suggesting that the trapping phenomenon is site specific on the SF_1. Less than stoichiometric trapping of ϵADP was observed in some of the cases at the 25-min termination point due to the slower kinetics of inactivation in the presence of ϵADP vs. ADP. In the case of DTNB-mediated disulfide formation, the time course of inactivation was sufficiently long (24 h) to permit significant leakage of trapped ϵADP.

It is known from previous work that MgADP markedly enhances the rate of inactivation of SF_1 ATPase activity by thiol cross-linking reagents (Wells et al., 1980a,c). Figure 1 shows that MgϵATP enhanced the rate of SF_1 inactivation by the Co^{II}/Co^{III}phen system. The limiting $t_{1/2}$ value of 4 min was significantly longer than the 1.5-min $t_{1/2}$ obtained under similar conditions using MgADP (Wells & Yount, 1979). This result may reflect a decreased reactivity of SH_2 in the presence of MgϵADP relative to that with MgADP as was suggested by the alkylation studies of Burke (1980). Figure 1 also shows that only stoichiometric amounts of ϵATP are needed to obtain the maximal rate of inactivation, further substantiating that ϵATP is acting at a single specific site.

Table II: Polarization Values of ϵADP Bound or Trapped on SF_1

additions	native SF_1	SF_1 + Mg-ϵADP treated with pPDM
none		0.323
2 mM EDTA	0.010	0.321
2.5 mM Mg^{2+}	0.320	
2.5 mM Mg^{2+} + 0.17 mM ATP	0.009	0.320
2.5 mM Mg^{2+} + 0.17 mM ADP	0.027	0.317
2.5 mM Mg^{2+} + 0.17 mM PP_i	0.006	0.315

[a] Conditions: measurements were made in KCl–Tris buffer, pH 8.0 at 6 °C. For native SF_1, ϵADP was added such that ϵADP/SF_1 = 0.10 to assure >95% of the ϵADP was bound to the SF_1. MgϵADP was trapped by stoichiometric addition of pPDM with respect to SF_1 (17 μM) and the reaction terminated after 25 min. λ_{ex} = 330 nm; λ_{em} = 410 nm. Slit widths were 4 nm. Polarization measurements were made 5 min after each specified addition and were corrected for light scattering and tryptophanyl emission contributions to the apparent fluorescence. Polarization of ϵADP in KCl–Tris buffer, pH 8.0 at 5 °C, was 0.004.

Competition experiments were carried out to demonstrate that the site of ϵADP trapping was the active site of SF_1. Table II shows that the polarization values of reversibly bound and pPDM-trapped ϵADP are identical (P = 0.32) whereas that of ϵADP in Tris–KCl buffer is nearly zero. This indicates that pPDM cross-linking does not alter the orientation or mobility of the ϵADP bound to SF_1. Similar results (data not shown) were obtained by using the cross-linking reagents oPDM and DTNB, showing that the spanning length of the thiol cross-linking reagent (5–9 and 2 Å, respectively) had no effect on the rotational dynamics of ϵADP bound to SF_1. Addition of ATP, ADP, or PP_i to SF_1·ϵADP decreased the polarization to a value similar to that seen for free ϵADP. This is in marked contrast to the result obtained for the ϵADP trapped on SF_1 by pPDM, in which the emission polarization remains essentially unchanged after these additions. This is further proof that the site at which ϵADP is bound and trapped is the active site of SF_1 and that there is no involvement of any possible secondary sites.

The fluorescence intensity of ϵADP is increased by 20 ± 2% upon binding to chymotryptic SF_1 as shown in Figure 2. The emission maximum of enzyme-bound ϵADP is also blue shifted by 7 nm with respect to ϵADP in Tris–KCl buffer, indicating that the active site is nonpolar with respect to the bulk solvent (Secrist et al., 1972; Spencer et al., 1974). Trapping of ϵADP by pPDM resulted in a further 24 ± 6% increase in fluorescence intensity but gave no change in the wavelength of the emission maximum. Absorption spectra of free, enzyme-bound, and trapped ϵADP show (see inset, Figure 2) that in the region used for excitation, 330 nm, the absorbance increases by 70% for bound and by a further 22% for trapped ϵADP. These absorption results are comparable to those obtained by Secrist et al. (1972) for ϵ-adenosine in dioxane and again indicate that the dielectric constant of the catalytic site is low at the purine binding site. The relative quantum yield of SF_1-bound vs. free ϵADP was 0.67 as calculated by eq 1 (Miller, 1981):

$$\frac{\Phi_b}{\Phi_f} = \frac{I_b A_f}{I_f A_b} \quad (1)$$

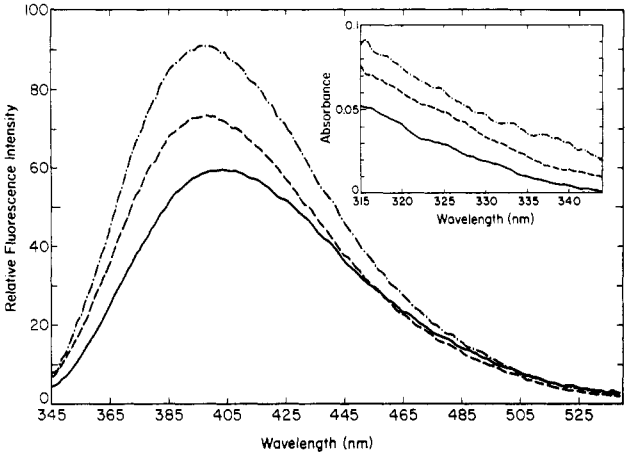

FIGURE 2: Corrected fluorescence emission spectra of 9.03 μM ϵADP in KCl–Tris buffer, pH 8.0, and 2 mM $MgCl_2$ (—), bound to 80.3 μM SF_1 (---), and trapped on SF_1 by reaction with 80.3 μM pPDM for 25 min (-·-). The excitation wavelength was 330 nm to minimize SF_1 light scattering and tryptophanyl emission contributions to the emission spectrum of bound or trapped etheno nucleotide. Such scattering as remained was computer subtracted out by use of the nucleotide-free SF_1 spectrum. All emission spectra were measured at 6 °C with the excitation polarizer set at 55° and the emission polarizer set at 0° to eliminate the effects (see text) of anisotropic rotation on the intensity of the bound and trapped fluorophore. (Inset) Absorption spectra of ϵADP (30.9 μM) in 2 mM $MgCl_2$ and KCl–Tris buffer, pH 8.0 (—), bound to 61.9 μM SF_1 (---), and trapped on SF_1 by reaction with 61.9 μM pPDM for 25 min (-·-). Spectra were recorded and stored on a Hewlett-Packard 9825A desktop computer interfaced to a Cary-14 spectrophotometer. Each spectrum is an average of three individual spectra ($\sigma \simeq 10\%$). The light scattering contribution of the SF_1 to the apparent absorbance of enzyme-bound or -trapped ϵADP was computer subtracted out. A small absorption contribution due to the pPDM thiol adduct was corrected by using a pPDM–glutathione control. All measurements were made at 6 °C.

where Φ represents quantum yields, I represents the integrated areas of the corrected fluorescence emission spectra, and A represents the absorbance values at the excitation wavelength of the bound (b) or free (f) fluorophore. The same result is obtained for the trapped ϵADP, since with the observed increase in emission intensity there is a corresponding increase in the absorbance at the excitation wavelength.

The linear polarization values of ϵADP bound on SF_1 as a function of wavelength are shown in Figure 3. The polarization varied significantly across the emission band, ranging in value from 0.44 at 345 nm to 0.22 at 500 nm. In contrast, the linear polarization spectrum of ϵAMP in propylene glycol did not vary appreciably from a value of 0.3 over the same wavelength region. Similar results have been obtained in linear polarization studies of ϵNAD^+ both in glycerol and bound to glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, or liver alcohol dehydrogenase (Gafni et al., 1979). The results in Figure 3 cannot be explained by the presence of free ϵADP since greater than 99% of the nucleotide is bound to SF_1 under these conditions. In addition, no photobleaching occurred, and successive polarization spectra were identical. Since the polarization of a fluorophore is inversely proportional to its lifetime, variation in the polarization could result if the bound fluorophore had multiple lifetimes each with different emission spectra. Another explanation is that the polarization properties of ϵADP depend on the vibrational levels of the ground and excited states, both of which may change on binding to the enzyme (Steinberg, 1975).

The variation in polarization at the emission maximum of ϵADP as a function of the ratio of nucleotide to SF_1 is shown in Figure 4. The ordinate intercept of this plot yields a limiting polarization value for ϵADP bound to SF_1 (P_b) of

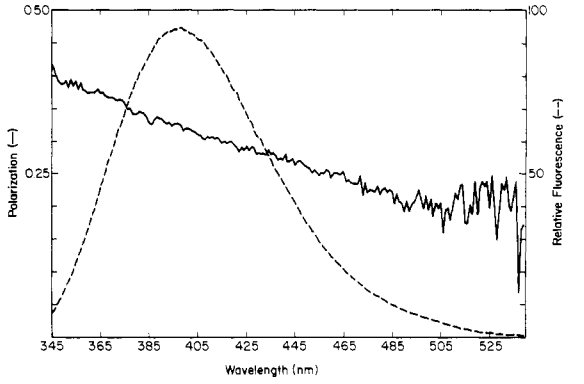

FIGURE 3: Linear polarization of fluorescence emission of 13.13 μM ϵADP bound to 174 μM SF_1 in KCl–Tris buffer, pH 8.0, and 2 mM $MgCl_2$, at 6 °C. By use of a $K_D = 1.83 \times 10^{-6}$ M (see Figure 4), >99% of the Mg-ϵADP is bound to the SF_1 so that the contribution of free Mg-ϵADP was negligible. The polarization spectrum of SF_1-bound Mg-ϵADP was obtained by measuring the emission spectra at polarizer settings (V,V), (H,V), (H,V), and (V,H) using a 330-nm excitation wavelength. Similar measurements were made on a nucleotide-free SF_1 solution at each polarizer setting and were computer subtracted from the comparable spectra given above. The polarization spectrum was generated from the stored spectra according to the relation $P = (I_{VV} - 2GI_{VH})/(I_{VV} + 2GI_{VH})$ where $G = I_{HV}/I_{HH}$.

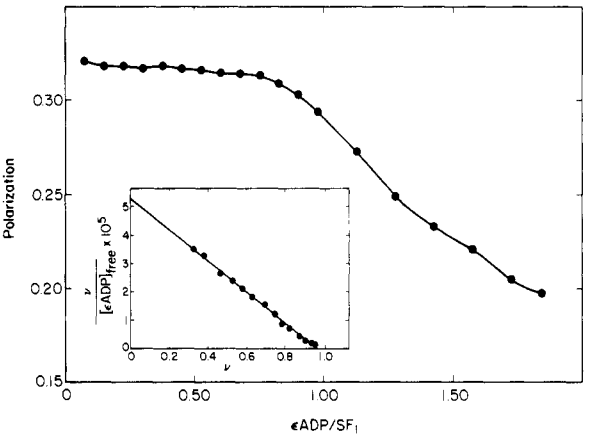

FIGURE 4: Titration of SF_1 with MgϵADP using emission polarization. SF_1 was 80.3 μM in KCl–Tris buffer, pH 8.0 at 6 °C. The excitation and emission wavelengths were 330 and 410 nm, respectively. All polarization measurements were corrected for SF_1 scatter contribution by subtraction of the intensity of an 80.3 μM SF_1 blank from that of the sample. The inset to the figure shows a Scatchard analysis of the above data. The dissociation constant under these conditions is 1.83×10^{-6} M, the stoichiometry is 0.96, and the correlation coefficient for the line is −0.99.

0.325. The same P_b value was obtained in a similar experiment using ϵAMP-PNP, showing that the presence of the γ-phosphate does not measurably effect either the conformation of SF_1 or the rotational dynamics of the bound etheno nucleotides. The binding constant of ϵADP to SF_1 under these conditions was determined by using the Weber (1952) equation as modified by Rajkowski & Cittanova (1981):

$$\frac{S}{(1-S)} = \frac{I_f(3-P_b)(P-P_f)}{I_b(3-P_f)(P_b-P)} \quad (2)$$

where S is the fraction of ligand bound, I is the emission intensity, P is the polarization, and the subscripts b and f denote bound and free fluorophore. A plot of data according to this equation (inset, Figure 4) yields an association constant of 5.5×10^5 M^{-1} with a stoichiometry of 0.96. This association constant is in close agreement with those obtained for ADP under similar conditions by Beinfeld & Martonosi (1975) and Yoshita & Morita (1975). Garland & Cheung (1976) used

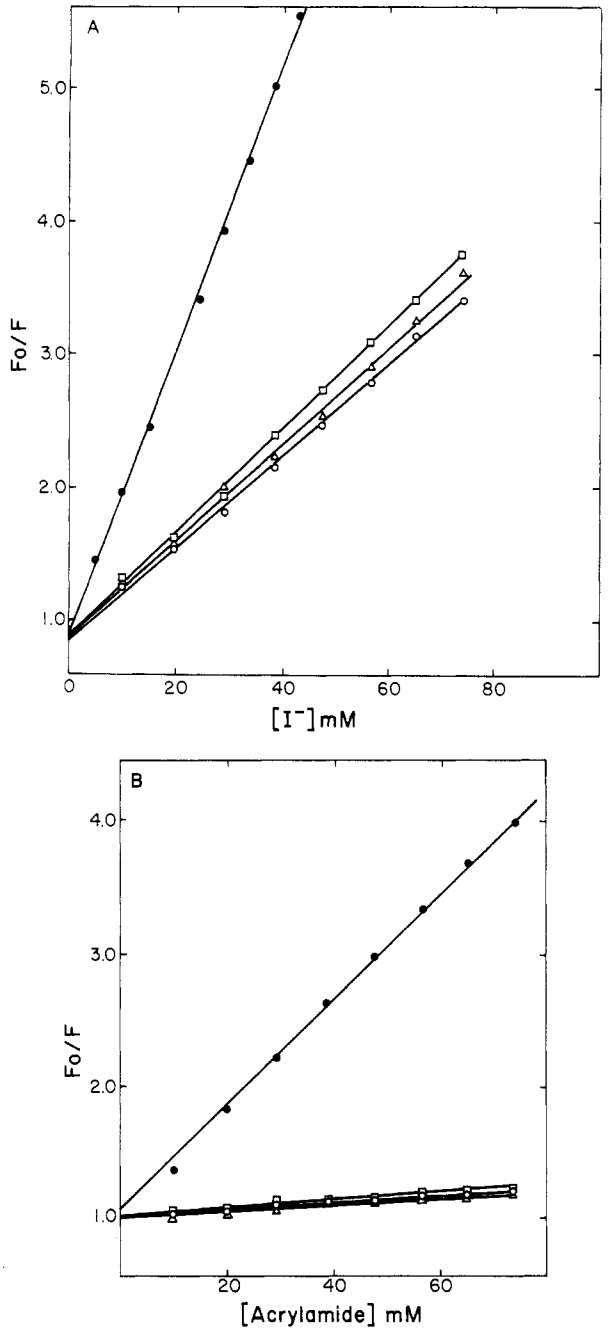

FIGURE 5: Stern–Volmer plots of iodide and acrylamide quenching of ϵADP free, bound, and trapped on SF_1. All measurements were made with 17 μM SF_1 in Tris–KCl buffer, pH 8.0 at 5 °C. (●) Unbound ϵADP; (□) Mg-ϵADP bound to SF_1 with [ϵADP]/[SF_1] = 0.2; (○) Mg-ϵADP trapped on SF_1 by pPDM; (△) Mg-ϵADP trapped on SF_1 by reaction with DTNB. Trapping procedures were as described under Materials and Methods. (A) Iodide quench curves; (B) acrylamide quench curves.

stopped-flow fluorescence and equilibrium dialysis to obtain somewhat weaker affinity constants, 7.4×10^5 and 8.0×10^5 M^{-1}, respectively, for ϵADP to SF_1 and HMM. It may be worth emphasizing that, in general, measuring changes in polarization is a more sensitive method for determination of a binding constant because the change in the observed parameter is typically greater than that observed in fluorescence intensity measurements.

Fluorescence quenching data for ϵADP unbound, bound reversibly, and trapped on SF_1 are shown in Figure 5. The linear nature of the data indicates that quenching by both iodide and acrylamide follow the equation of Stern & Volmer (1919) as modified by Lehrer & Leavis (1978):

$$F_0/F = 1 + K_{sv}[Q] = 1 + k_q\tau_0[Q] \quad (3)$$

where K_{sv} is the Stern–Volmer constant, k_q is the quenching rate constant, τ_0 is the fluorescence lifetime of the fluorophore in the absence of the solute quencher, and [Q] is the solute quencher concentration. This indicates the quenching mechanism is dynamic or collisional. The cross-linking reagents used in these studies, pPDM and DTNB, result in thiol spanning lengths of 12 and 2 Å, respectively (Wells et al., 1980c). It is evident from the slopes of the lines presented in Figure 5A that reversibly bound and trapped ϵADP are equally accessible to iodide and that this accessibility, while lower than that for unbound ϵADP, is still appreciable. The spanning length of the cross-linking reagent used has little effect upon the solute quencher accessibility and gave a result identical with that for fluorophore bound reversibly to SF_1. The same general results were obtained for the acrylamide quenching studies (Figure 5B). However, acrylamide was much less effective than iodide as a quenching agent for ϵADP either free, bound reversibly, or trapped on SF_1.

Effects of quenching agents on ϵADP lifetimes and fluorescence intensities together with the Stern–Volmer quenching constants (calculated from Figure 5) are presented in Table III. That the ratio of the fluorescence intensities (F_0/F) is equal to the ratio of the lifetimes (τ_0/τ) in the absence and the presence of the quencher is more direct evidence that the quenching mechanism is dynamic. The k_q value determined for the iodide interaction with unbound ϵADP (3.9×10^9 M^{-1} s^{-1}) is lower than the theoretical diffusion limited rate constant at 6 °C (6.29×10^9 M^{-1} s^{-1}).[2] This observation may be explained by the electrostatic repulsive force between the I^- and the negatively charged phosphates of ϵADP as pointed out by Ando & Asai (1980). The quenching rate constant for iodide decreases by a factor of 3 upon binding or trapping of ϵADP to SF_1, indicating that there is partial shielding of the purine portion of the nucleotide at the catalytic site. The quenching rate constant for acrylamide on unbound ϵADP is significantly lower than that obtained for iodide, and there appeared to be some static quenching involved since $F_0/F > \tau_0/\tau$. In contrast to the result for iodide, the quenching rate constant for acrylamide decreased by an order of magnitude upon binding or trapping of ϵADP to SF_1. Ando et al. (1982) have recently used this difference in acrylamide quenching to distinguish enzyme bound from free ϵADP. The difference in acrylamide and iodide accessibility to bound or trapped ϵADP is not attributable to charge since acrylamide is neutral. Because the largest dimension of acrylamide (7.2 Å) is larger than the diameter of I^- (4.4 Å), the lower accessibility of acrylamide may be due to steric constraints at the catalytic site. Alternatively, acrylamide may not achieve as many effectively oriented collisions with bound ϵADP as I^- because it is not a spherically symmetric quenching species.

It was of interest to investigate the polarization value of trapped ϵADP relative to the thiol spanning length of the cross-linking reagent. While no correlation in these parameters was found, it was observed that with time the polarization values decreased. With chromophoric cross-linking agents the total fluorescence increase paralleled the decrease in polarization. Figure 6 shows such a result for the F_2DPS cross-linked system. These results indicate that ϵADP leaks from

[2] This value was calculated from the value given in Ando & Asai (1980) by correcting for the difference in temperature and assuming that the equivalent conductance of iodide does not vary appreciably in the temperature range 6–25 °C.

Table III: Fluorescence Lifetimes and Rate Constants for Solute Quenching of ϵADP Fluorescence[a]

etheno nucleotide system	quencher	quencher concn (mM)	F_0/F	fluorescence lifetime (ns)	τ_0/τ	K_{sv} (M^{-1})	k_q (M^{-1} s^{-1}) × 10^{-9}
(1) Mg-ϵADP in Tris–KCl	KI	0		26.6			
		65	4.47	5.5	4.81	106 ± 1.5	3.98
	acrylamide	0		27.4			
		65	3.68	10.2	2.68	38.9 ± 0.7	1.44
(2) Mg-ϵADP·SF_1[b]	KI	0		20.5			
		10	1.52	13.7	1.49	36.9 ± 1.2	1.39
	acrylamide	0		20.5			
		10	1.07	18.5	1.11	3.1 ± 0.1	0.15
(3) Mg-ϵADP·SF_1 (pPDM cross-linked)	KI	0		21.4			
		10	1.28	16.9	1.27	35.9 + 0.9	1.35
	acrylamide	0		21.6			
		10	1.03	21.1	1.02	2.8 ± 0.2	0.13
(4) Mg-ϵADP·SF_1 (–S–S–) by DTNB	KI	0		20.1			
		10	1.26	16.1	1.25	34.2 ± 0.6	1.28
	acrylamide	0		20.2			
		10	1.04	19.8	1.02	2.9 ± 0.1	0.14

[a] The rate constants for quenching of ϵADP fluorescence were determined by use of eq 3. Stern–Volmer quenching constants, K_{sv}, were obtained from slopes determined by linear regression analysis of the data shown in Figure 5. F_0/F and τ_0/τ are the ratios of the fluorescence intensities and lifetimes in the absence and presence of the solute quenching agent, respectively. Lifetimes were generally measured at a modulation frequency of 6 MHz. τ modulation and τ phase values typically agreed to within 1 ns, indicating that the systems were essentially homogeneous. [b] Mg-ϵADP/SF1 = 0.1 so that at [SF_1] = 17 μM >95% of the ϵADP is bound to SF_1.

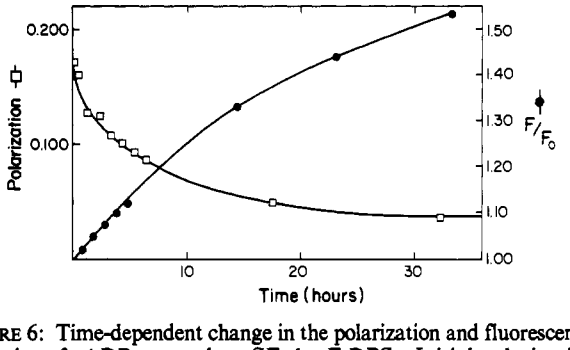

FIGURE 6: Time-dependent change in the polarization and fluorescence intensity of ϵADP trapped on SF_1 by F_2DPS. Initial polarization measurements were made 30 s after collection of the purified SF_1 Mg-ϵADP complex from a Sephadex G-25 column (Pharmacia PD-10). All cross-linked derivatives were prepared as described under Materials and Methods from the same SF_1 preparation. Measurements were made at 5 °C on an MPF-3L spectrofluorometer using an excitation wavelength of 320 nm.

the active site where its total fluorescence was quenched either by trivial transfer or by Förster energy transfer (Förster, 1959). To distinguish between these two possibilities, we studied the lifetime of ϵADP trapped on SF_1 by a variety of cross-linking agents. Table IV shows that while ϵADP exhibits a decreased lifetime on binding to SF_1, no further change occurs upon cross-linking by NDM, pPDM, oPDM, or DTNB. These cross-linking reagents are nonchromophoric and give thiol to thiol spanning distances ranging from 14 to 2 Å. These results indicate the microenvironment of the purine binding portion of the active site is unperturbed by cross-linking or by variation in the thiol spanning distance. On the other hand, decreased lifetimes were observed (Table IV) after cross-linking with F_2DPS, F_2DNB, or Co^{III}phen. The thiol adducts of these three reagents all have absorption spectra that overlap the emission spectrum of bound ϵADP. The spectral overlap between ϵADP and these cross-linking agents, the absence of an effect on the active site by cross-linking, and the observed decreased lifetimes all indicated that Förster energy transfer and not trivial transfer was taking place. The observation that τ modulation was greater than τ phase for the chromophoric cross-linking agents (Table IV) indicates lifetime heterogeneity (Spencer & Weber, 1969). This heterogeneity is best explained by leakage of ϵADP from the active site which would give rise to the presence of both free and trapped forms of the nucleotide. The lifetimes of the trapped and free ϵADP have been

Table IV: Fluorescence Lifetimes of ϵADP Trapped on SF_1 by Various Cross-Linking Reagents[a]

cross-linking reagent	τ phase	τ modulation
pPDM	21.6	21.8
oPDM	21.2	20.8
DTNB	20.1	20.7
NDM	20.5	21.6
F_2DPS	11.3	16.2
F_2DNB	7.77	11.9
Co^{II}phen	6.8	7.8

[a] [SF1] = 17 μM, pH 8.0, 4 °C. oPDM and NDM system lifetimes were measured with the modulation frequency set at 6 MHz. F_2DPS, F_2DNB, and Co^{II}phen system lifetimes were measured at 30 MHz. These values were not corrected for the wavelength-dependent response of the photomultiplier tubes.

Table V: Temperature Dependence of k_{off} of Trapped ϵADP on SF_1 by pPDM[a]

T (°C)	$10^3/T$	log k_{off}	k_{off} (min^{-1})	$t_{1/2}$ (min)
30	3.298	−1.57	0.038	18.2
25	3.354	−1.78	0.017	41.8
20	3.411	−2.29	0.005	135
15	3.470	−2.64	0.002	300
10	3.532	−3.20	0.0006	1080
5	3.595	−3.88	0.0001	5330

[a] SF_1 (17 μM) was inactivated with 22 μM pPDM in the presence of 0.1 mM ϵADP and 2 mM Mg^{2+}. The enzyme was purified by $(NH_4)_2SO_4$ precipitation and gel filtration as described under Materials and Methods. The polarization of the ϵADP was measured as a function of time, beginning within 30 s of the elution of pPDM·Mg^{2+}-ϵADP·SF_1 from the gel filtration column.

measured and used to calculate the distance between the cross-linked thiols and the active site (Perkins et al., 1980, 1984).

Examination of the polarization of the trapped ϵADP showed that P decreased with time. Parallel studies of the amount of metal ion trapped showed a similar decrease with time (data not shown). This suggested that ϵADP leaked out of the catalytic site of SF_1 according to the equation

$$\text{pPDM–SF}_1\cdot\epsilon\text{ADP-Mg} \overset{k_{off}}{\rightleftharpoons} \text{pPDM–SF}_1 + \epsilon\text{ADP-Mg} \quad (4)$$

The leaking appears to be irreversible because the polarization value at infinite time approximates that of the unbound fluorophore (P_f). Use of eq 2 permits calculation of the amount of the nucleotide bound at any given time by mea-

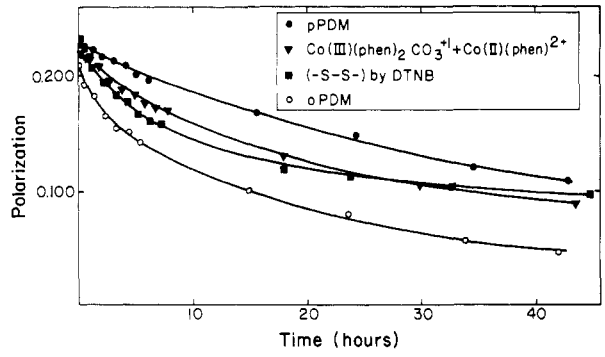

FIGURE 7: Measurement of the time-dependent change in polarization of ϵADP trapped on SF_1 by a variety of cross-linking reagents. For conditions refer to Figure 6. Measurements were made at 5 °C on an MPF-3L spectrofluorometer using film-type polarizers at an excitation wavelength of 320 nm. Under these conditions the P_b is 0.21. Cross-linking reagents used: (●) pPDM; (▼) Co^{II}phen; (■) (-S-S-) by DTNB; (○) oPDM.

surement of the polarization. This in turn permits calculation of k_{off}, assuming that the leaking process is either irreversible or that the K_{eq} (eq 4) is very large. Table V shows the effect of temperature on the first-order off-rate of trapped ϵADP as detemined by measuring its time-dependent polarization at various temperatures. An Arrhenius plot of these data was linear (correlation coefficient = 0.99) with an E_a = 39.6 kJ mol^{-1}. The relatively high off-rates at higher temperatures indicate that prolonged studies employing trapped ϵADP (e.g., Förster energy transfer experiments) must be performed at 0–5 °C.

The time dependence of the polarization of ϵADP at 6 °C trapped by various cross-linking agents is shown in Figure 7. Surprisingly, there was no correlation between the stability of trapped ϵADP and the thiol spanning length of each cross-linking agent. For example, ϵADP trapped by disulfide bond formation (DTNB, 2 Å) or cobalt chelation (Co^{III}phen, 3–5 Å) was intermediate in stability with respect to ϵADP bound to SF_1 modified with pPDM (12 Å) or oPDM (5–9 Å). As mentioned above, one of the cross-linking agents studied, F_2DPS, has spectral overlap with the emission of ϵADP after its reaction with SF_1. A parallel study of ϵADP stability and emission intensity shows that, as ϵADP leaked out of the catalytic site, its fluorescence intensity increased. This is a further indication that Förster energy transfer was occurring between ϵADP (donor) and the dithiol adduct of F_2DPS (acceptor).

Discussion

These studies demonstrate that a fluorescent analogue of ADP, ϵADP, can be trapped at the catalytic site of SF_1. Like ADP (Wells & Yount, 1979), ϵADP markedly enhanced the inactivation of SF_1 by thiol cross-linking reagents and was trapped in nearly stoichiometric quantities as the metal–nucleotide complex. The rate of inactivation of both the K^+- and Ca^{2+}-ATPase was, however, slower with ϵADP than observed with ADP. This suggests that the enzyme conformation about the critical thiols, SH_1 and SH_2, is slightly different for the two complexes, a result which must be considered when intrinsic fluorescence studies of the SF_1 ATPase are interpreted using ϵATP (Garland & Cheung, 1976, 1979).

It has been suggested that thiol cross-linking reagents trap nucleotides at the SF_1 catalytic site by directly locking shut a nucleotide cleft (Wells & Yount, 1979). The use of fluorescent ATP analogues provides a unique way to test such a suggestion. For example, predictions of such a model are that, as the resultant thiol spanning length is descreased, (a) the steric constraints on the nucleotide at the catalytic site should increase, possibly giving rise to an increase in polarization of the trapped fluorophore, (b) the accessibility of the trapped nucleotide to solute quenching agents should decrease, and (c) the stability of the trapped nucleotide should increase. We observed differences in neither the polarization nor the solute quencher accessibility of trapped ϵADP as a function of thiol spanning length. While the stability of trapped ϵADP differed for the different cross-linking reagents, it did not do so in the predicted manner. For example, ϵADP trapped by pPDM (sulfur to sulfur distance = 12 Å) was found to be more stable than ϵADP trapped by DTNB mediated disulfide formation (sulfur to sulfur distance = 2 Å). It is likely, therefore, that the cross-linking agents act indirectly to stabilize nucleotide binding rather than directly locking closed a jawlike active site. Such an indirect sterically contrained model has recently been used to explain nucleotide-enhanced Förster energy transfer between a donor on SH_1 and an acceptor on SH_2 (Dalbey et al., 1983a). A similar indirect model was previously proposed by Yamamoto & Sekine (1982) on the basis of studies in which they were able to trap an ATP analogue after SH_1 and SH_2 had presumably been cross-linked by pPDM in the absence of nucleotide.

Evidence from these studies that the active site of myosin is buried is indicated by (i) the observation of trapping at the active site, (ii) the blue shift in the emission maximum of bound vs. free ϵADP, signifying the purine binding portion has a low dielectric constant,[3] (iii) the decreased solute quencher accessibility to bound or trapped fluorophore, and (iv) the observation that, once the nucleotide leaks out of the catalytic site, it does not appreciably bind again. The latter observation may be explained by the relative dilution of the nucleotide that takes place when it escapes from the small volume of the catalytic site into the much larger volume of the bulk solvent. That both the on-rate and off-rate of nucleotide to the catalytic site are decreased by thiol cross-linking agents is well documented (Wells & Yount, 1982). The temperature dependence of k_{off} in the present studies indicates that although thiol cross-linking introduces steric constraint on the active site, there is still sufficient conformational flexibility to permit nucleotide release. Such flexibility is also borne out by the increased off-rate of trapped nucleotide with actin binding (Perkins et al., 1981) and increasing ionic strength (Dalbey et al., 1983b).

Other workers have reported that there is no change (Willick et al., 1973) or a decrease (Onishi et al., 1973; Yanagida, 1981) in the emission intensity of ϵADP upon binding to the myosin active site. The magnitude of the reported decrease in fluorescence intensity increases with temperature (Yanagida, 1981). For systems having a fluorescent ligand bound to a large, anisotropically rotating macromolecule such as myosin or SF_1, the measured emission intensity is markedly dependent upon the polarizer settings used for making the measurements (Weber & Teale, 1957). Discrepancies in the observed change in intensity of ϵADP upon binding to the myosin catalytic site such as those mentioned above will almost always result when the polarization settings of the detector system are not carefully

[3] This result was verified in SF_1 binding studies using ATP analogues having spectra more sensitive to changes in medium dielectric constant. The chromophoric ADP analogue *N*-(4-azido-2-nitroanilino)ethyl diphosphate shows an absorption blue shift consistent with a dielectric constant of 45 (K. Nakamaye, J. Wells, R. Bridenbaugh, and R. Yount, unpublished results). 1,N^6-ϵ-Aza-ADP shows a very large emission blue shift on binding to SF_1 or HMM (Miyata & Asai, 1982) that is compatible with a dielectric constant similar to that of dioxane (W. Perkins, unpublished results).

controlled. Using no polarizers makes intensity measurements subject to the uncontrolled degree of polarization in exciting light introduced by the excitation monochromator. In addition, the emission monochromator may pass light of a particular polarization more efficiently than others. For a vertically oriented emission polarizer and right angle viewing, the fluorescence intensity is unbiased by polarization if it is excited with an excitation polarizer set at 54.75° or 125.25° with respect to the vertical axis (Spencer & Weber, 1970; Cehelnik et al., 1975). An alternative procedure for obtaining an unbiased intensity measurement is that of Azumi & McGlynn (1962) in which polarizers are used to isolate the vertically and horizontally polarized components of the exciting light and the fluorescence emission. We employed the latter technique to verify the validity of the previously described procedure. Illustrating the variability in intensity changes expected when the polarizer settings are not carefully controlled, we observed a decrease in the emission intensity (7 ± 1%) of ϵADP when we used a vertically oriented excitation polarizer and no emission polarizer. When, however, measurements were made so that the effects of polarization on emission intensity were eliminated, we observed the reported increase in the intensity upon binding of ϵADP to SF_1. The same increase was observed for ϵADP and ϵAMP-PNP, which shows that different conformations of the enzyme have no effect on the fluorescence properties of etheno nucleotides.

Though the emission intensity of SF_1-bound ϵADP increases with respect to the nonbound nucleotide, its relative quantum yield decreases (cf. Results), which is attributable to the decreased dielectric constant (Secrist et al., 1972) around the purine binding portion of the catalytic site. The fluorescence lifetime of SF_1-bound ϵADP also decreased, a result expected for ethenonucleotides in a nonpolar environment (Spencer et al., 1974). Comparison of bound and trapped ϵADP shows that they are identical in polarization, emission maximum, quantum yield, solute quencher accessibility, and fluorescence lifetime. The only difference between bound and trapped ϵADP we have observed is an increase in absorbance at 330 nm which is paralleled by a corresponding increase in emission intensity for the fluorophore trapped on SF_1. We conclude, therefore, that cross-linking and trapping only minimally perturb the microenvironment at the catalytic site and that the trapped nucleotide is a suitable model for reversibly bound nucleotide in future studies.

The steady-state polarization of enzyme-bound fluorophore can be used to calculate the rotational relaxation time of the nucleotide according to the relation (Perrin, 1934)

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_h}\right) \qquad (5)$$

where P_0 is the intrinsic polarization of the fluorophore, τ is the lifetime of enzyme-bound fluorophore, and ρ_h is the harmonic mean rotational relaxation time. Using P_0 equal to 0.38 (Secrist et al., 1972) and P set equal to P_b (0.325), a rotational relaxation time of 315 ns was obtained (the rotational correlation time, t_c^{-1}, is 105 ns). This is significantly lower than the values obtained by Mendelson et al. (1973) or Thomas (1978) using fluorescence depolarization and EPR techniques for reporter groups on SH_1 of SF_1. It is, however, similar to the result obtained by Shriver & Sykes (1981) using ^{31}P NMR to study ADP bound to SF_1, suggesting that the alignment of ϵADP emission dipole moment is nearly the same as the principal axis of the β-phosphate chemical shift tensor.

Assignment of the emission dipole moment orientation with respect to the long axis of SF_1 is problematic. The polarization of ϵADP bound to SF_1 is known to vary across the emission band, whereas the polarization of etheno nucleotide derivatives does not vary appreciably in highly viscous solvents (Gafni & Steinberg, 1979). It is not possible, at present, to decide upon the appropriate P_b value for such a system. We have chosen to use the polarization at the emission maximum as an operationally defined P_b. Another complication arises from the absence of a simple hydrodynamic model for SF_1. It is probable, therefore, either that the ϵADP emission dipole moment is along an axis having a lower rotational relaxation time than that obtained from reporter groups on SH-1 or that the nucleotide retains some rotational freedom at the catalytic site.

Finally, the observation that the lifetime of ϵADP decreases when it is trapped by chromophoric cross-linking agents as F_2DPS or Co^{III}phen suggests that the active site is within Förster energy transfer distance of SH_1 and SH_2. This unique situation in which a donor and acceptor are introduced onto a macromolecule in a single covalent modification has been used to determine the distance between the active site and the cross-linked SH_1 and SH_2 (Perkins et al., 1984).

Acknowledgments

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Registry No. ϵADP, 38806-39-2; Co(II)phen, 29020-64-2; pPDM, 3278-31-7; σPDM, 13118-04-2; NDM, 58487-16-4; F_2DPS, 312-30-1; DTNB, 69-78-3; MgϵADP, 70824-96-3; F_2DNB, 327-92-4; [Co(III)(phen)$_2CO_3$]$^+$, 35672-83-4; ATPase, 9000-83-3; ϵAMP-PNP, 78368-53-3; iodide, 20461-54-5; acrylamide, 79-06-1.

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