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Solvent Accessibility of the Adenosine 5′-Triphosphate Catalytic Site of Sarcoplasmic Reticulum CaATPase†

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Received June 17, 1985

ABSTRACT: The CaATPase of rabbit skeletal sarcoplasmic reticulum was labeled at or near the ATP catalytic site with fluoresceinyl isothiocyanate (FITC), and the accessibility of the attached probe to the bulk solvent was determined by I^- quenching of its fluorescence. The quenching of free FITC was also measured. In both cases, the quenching was of the Stern–Volmer type and collisional quenching rate constants were obtained over the pH range 5–8 in the presence of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid and with added Ca^{2+}, vanadate, or phosphate. The fluorescence intensity and susceptibility to quenching by I^- of free FITC were insensitive to the added ligands. In all cases, the intensity decreased with pH, as predicted from the known properties of FITC mono- and dianions. The collisional quenching rate constants increased at lower pH, as expected for I^- quenching of a molecule with decreasing negative charge due to protonation. When FITC was attached to the CaATPase, the FITC fluorescence intensity and I^- collisional quenching rate constants were sensitive to ligand binding as well as pH. The changes in fluorescence intensity with acidity, when compared to the results for free FITC, indicated the pK_a of the FITC was reduced 0.6 unit when it was attached to the CaATPase. Excited-state lifetime measurements indicated that ligand effects at constant pH were not due to protonation-induced changes in FITC quantum yield but to conformational changes of the CaATPase. The ligand-induced changes in the collisional quenching rate constants appeared to be due to changes in steric hindrance to I^- colliding with bound FITC rather than changes in local charge near the probe. At all pH values, the hindrance to I^- quenching of the fluorescence of the FITC at the ATP binding site was vanadoenzyme < enzyme < phosphoenzyme. The effect of Ca^{2+} binding to the CaATPase was to decrease the hindrance. The lower the pH, the greater was the decrease caused by Ca^{2+} binding. At pH 6.0, the transition from E–P to ECa_2 was consistent with an increase of 48% in the accessibility of the ATP binding site to the bulk solvent.

The CaATPase of skeletal muscle sarcoplasmic reticulum (SR)[1] uses the energy of MgATP binding and hydrolysis to move two bound calcium ions from the cytosolic side into the lumen of the membrane (Inesi, 1981; de Meis, 1981; Hasselbach & Oetliker, 1983). This interaction between the ATP binding site and the Ca^{2+} binding sites also is apparent from

† This work was supported by grants from NIH (AM 25177 and AM00509), NSF (CDP-7923045), and the California Affiliate of the American Heart Association with contributions from the San Francisco Chapter.

[1] Abbreviations: SR, sarcoplasmic reticulum; CaATPase, (Ca^{2+},-Mg^{2+}) adenosinetriphosphatase from skeletal muscle SR; K_Q, slope of the Stern–Volmer plot; k_Q, Stern–Volmer collisional quenching rate constant; τ, excited-state lifetime; *q*, quantum yield; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FITC, fluoresceinyl isothiocyanate.

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the 20-fold increase in the rate of ATP hydrolysis due to Ca^{2+} binding. Beyond these striking indications of interactions between the ATP hydrolyzing site and the high-affinity Ca^{2+} binding sites, few details of the nature of the energy-transducing interaction are known. Recently, it was shown that MgATP binding accelerates the binding of Ca^{2+} at its high-affinity binding sites (Stahl & Jencks, 1984). It also appears that the ATP and Ca^{2+} sites are not contiguous (Highsmith & Murphy, 1984; Scott, 1984), suggesting that ligand binding at one site is inducing a conformational change at a distant site. Mechanisms of transmitting structural changes from one binding site, through protein, to a second site may be universal for energy-transducing enzymes (Morales & Botts, 1979; Tanford, 1983). The experiments described here are an attempt to obtain more information about the interactions between the ligand binding sites of the SR CaATPase.

A covalently attached fluorescent probe that can be stoichiometrically and specifically placed at or near the ATP catalytic site of SR CaATPase is fluoresceinyl isothiocyanate. Pick and Bassilian (1981; Pick, 1981, 1982) have shown that FITC binds at the ATP site and its fluorescence intensity is sensitive to the binding of other ligands. The location of the attached FITC has been assigned to Lys-514 (MacLennan et al., 1985). There are several reasons to think that FITC is attached at the adenine binding subsite of the ATP catalytic site. The structure of the fluorescein has dimensions similar to those of adenine. ATP is an inhibitor of FITC incorporation (Pick & Bassilian, 1981; Highsmith, 1984). Likewise, CaATPase with FITC attached does not bind ATP (Andersen et al., 1982; Clore et al., 1982) or ATP analogues (Coll & Murphy, 1984; Nakamoto & Inesi, 1984) at the catalytic site. The stoichiometry of FITC binding that gives 100% inhibition of ATPase activity is very near that of maximum phosphorylation of the CaATPase (Mitchinson et al., 1982; Highsmith, 1984). However, the FITC-CaATPase is still active for smaller substrates that do not need the adenine binding subsite to bind; they can phosphorylate the modified enzyme and cause it to transport calcium (Pick & Bassilian, 1981). Although it cannot be rigorously excluded that FITC is bound elsewhere and merely affects the ATP site, these results suggest that FITC is a suitable probe for the ATP catalytic site and perhaps specifically for the adenine subsite.

In the experiments described here, a method developed by Lehrer (1971) was used to quantitate the accessibility of FITC at the ATP binding site to the bulk solvent. The effects of Ca^{2+}, phosphate, and vanadate on the Stern–Volmer quenching by I^- of FITC fluorescence were measured for FITC and CaATPase-FITC in the pH range 5–8. The major purpose of these measurements was to quantitate structural changes at the ATP site that are caused by ligand binding at other sites. Iodide was chosen because it is relatively small and can go where the bulk solvent can and because it is charged and is known not to penetrate into the protein interior (Eftink & Ghiron, 1977; Lehrer, 1971). It should be noted that there is also interest in the degree of solvent accessibility to the ATP binding site in connection with the contribution of substrate desolvation to the mechanism of ATP hydrolysis by SR CaATPase (de Meis et al., 1980).

Experimental Procedures

Sarcoplasmic reticulum vesicles were prepared from hind leg muscle obtained from New Zealand albino rabbits (Eletr & Inesi, 1972) and were stored at 0 °C in 30% (by weight) sucrose solutions for up to 3 days before use. Assayed by a phosphomolybdate method (Murphy, 1981), the calcium-activated ATPase activity at 37 °C was in the range of 6–10 μmol of P_i min^{-1} (mg of SR protein)$^{-1}$ and declined by less than 5% during the storage period.

Fluoresceinyl isothiocyanate labeling at the ATP binding site at slightly alkaline pH (7.6) has been described (Highsmith, 1984). The labeled vesicles were separated from the free FITC by size-exclusion chromatography using Bio-Gel P-10. The degree of labeling was assessed by the loss of ATPase activity (Andersen et al., 1982; Highsmith, 1984). All measurements involving FITC-labeled SR were made with samples that were labeled and purified on the same day as the measurement.

Steady-state fluorescence intensity measurements were made on a Perkin-Elmer MPF-44B fluorospectrophotometer. Fluorescence lifetime measurements were made with a laboratory-built time-resolved instrument using a free running hydrogen flash lamp and photon-counting detection of the fluorescene decay (Torgerson, 1984). Stern–Volmer quenching of fluorescence was analyzed by lifetime (Vaughan & Weber, 1970) and steady-state (Lehrer, 1971) methods. Values given for fluorescence intensity and Stern–Volmer slopes are averages of measurements on at least three samples and have uncertainties of less than ±12%. Data have been combined from six preparations of SR.

Results and Discussion

Iodide Interactions with CaATPase-FITC. The CaATPase of sarcoplasmic reticulum vesicles was specifically labeled at the nucleotide binding site with FITC at pH 7.6 in 100 mM KCl, 5 mM $MgCl_2$, 20 mM MOPS, 1 mM EGTA, and 0.1 mM $CaCl_2$ and purified by size-exclusion chromatography (Highsmith, 1984). The degree of labeling was varied between 1 and 5 nmol of FITC attached per milligram of SR protein by varying the [FITC] in the incubation mixture. Addition of I^- to solutions containing CaATPase-FITC caused a decrease in steady-state fluorescence intensity, F, which gave a linear Stern–Volmer plot in the 0–40 mM I^- range (Figure 1). Slopes, K_Q, were determined from these data with

$$F_0/F = 1 + K_Q[I^-]$$

In solutions containing Ca^{2+}, the slope was 4.1 ± 0.5 M^{-1}, independent of the degree of labeling. Parallel measurements of excited-state lifetimes for solutions of CaATPase-FITC containing iodide gave the same slope, indicating that the mechanism of quenching is collisional (Vaughan & Weber, 1970) with no apparent ground-state contributions (Figure 1). All the decays were well fit by a single exponential. The observed excited-state lifetime, without quenching, was 4.09 ± 0.03 ns. Thus, the rate constant for quenching by I^- at 25 °C and pH 7.0 is $k_Q = K_Q\tau^{-1} = 1.0 \times 10^9$ M^{-1} s^{-1} for CaATPase-FITC, and the mechanism is collisional with negligible dark complex contributions.

The dependency of the slope for Stern–Volmer quenching on the temperature and viscosity was determined for FITC and CaATPase-FITC (Figure 2). The free FITC is more readily quenched, and the changes with T/η are as expected for a collisional mechanism. The data are consistent with an extrapolation through the origin. The dependency of K_Q on T/η for CaATPase-FITC supports a collisional mechanism, as demonstrated by the lifetime measurements in that K_Q decreases linearly with T/η. However, the extrapolated value of $K_Q = 0$ for T/η near 100 instead of 0 K/cP is unusual and suggests that the attached FITC is protected from I^- quenching.

The high concentrations of I^- used to obtain the collisional rate constants did not affect the CaATPase activity. The presence of both iodide and phosphate did greatly enhance the

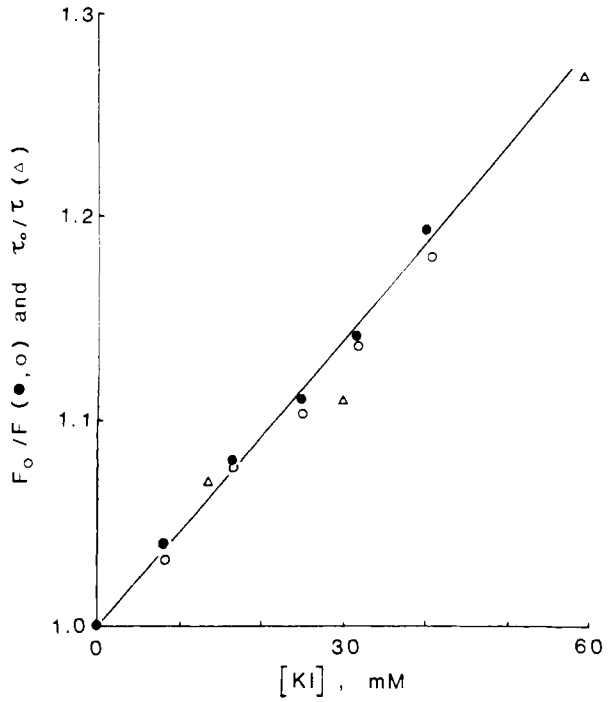

FIGURE 1: Collisional quenching of CaATPase-FITC by iodide. Vesicular SR CaATPase, labeled with FITC, was 0.05 mg of protein/mL in 100 mM KCl, 20 mM MOPS (pH 7.0), and 5 mM $MgCl_2$ at 25 °C. Stern–Volmer plots were made with data from steady-state fluorescence intensities (○ and ●) or from excited-state lifetimes (△). The ratio of nanomoles of FITC per milligram of SR protein was 1.1 (○), 4.1 (△), and 4.9 (●). KI stock solutions were 2.5 M and contained 1×10^{-4} M sodium thiosulfate.

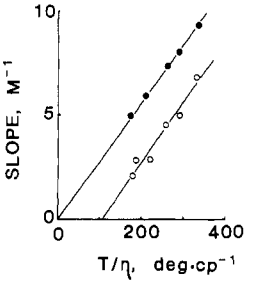

FIGURE 2: Viscosity dependence of I^- quenching of FITC and CaATPase-FITC. The slopes for SternVolmer quencing of FITC (●) and vesicular CaATPase-FITC (○) are plotted as a function of temperature and viscosity. In each case, the solutions were 75 nM FITC, 100 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole (pH 7.0), 1 mM EGTA, and 1 mM $CaCl_2$. The temperature was measured in the cuvette, and the viscosity was taken as that of water at that temperature.

absorbance of the phosphomolybdate assay solution, giving the appearance of activation of activity by I^-. However, when this was corrected, by doing controls with I^- added to the assay solution instead of the reaction mixture, the true activity did not change within experimental error for $[I^-]$ up to 40 mM. This result suggests that I^- is not binding to the CaATPase. It appears that the phosphomolybdate assay sensitivity could be increased by including iodide in the quench reaction.

Ligand and pH Effects on FITC Quenching. In order to resolve the effects of pH changes and of ligands directly on FITC from their effects on the CaATPase-FITC, the quenching of FITC fluorescence by I^- was investigated in the pH range 5–8. Fluorescein has a quinonoid structure with a pK_a near 6.7 for the transition between the dianion and the monoanion (Leonardt et al., 1971), so two species coexist in the physiological pH range. The quantum yields of the dianion and monoanion are 0.85 and 0.35, respectively (Parker & Rees,

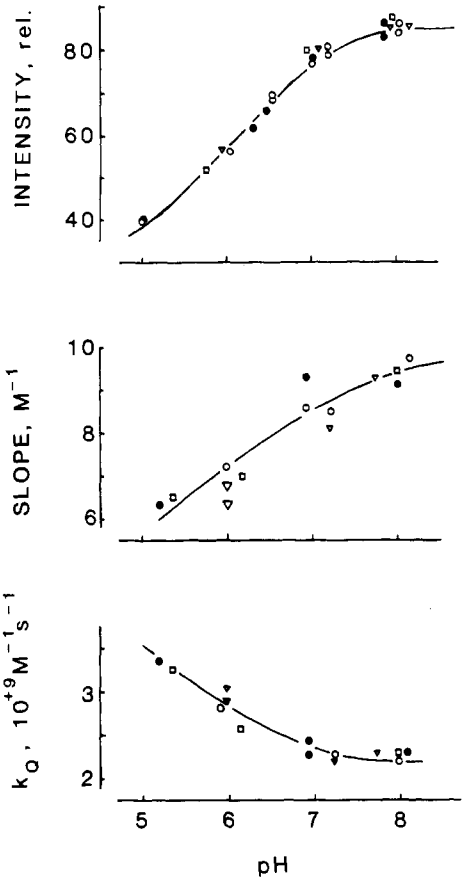

FIGURE 3: pH dependence of iodide quenching of FITC. (A) Intensities of FITC fluorescence for 200 nM FITC in 100 mM KCl, 5 mM $MgCl_2$, 20 mM MOPS, and 1 mM EGTA including no addition (○), 1.1 mM $CaCl_2$ (●), 50 mM phosphate (□), or 25 μM vanadate (▽) at the pH indicated. (B) Stern–Volmer quenching slopes obtained from data as shown in Figure 1 for added I^- up to 38 mM. Solution conditions and symbols are as in panel A. (C) Collisional quenching constants were calculated with the slopes given in panel B, and the effective excited-state lifetime was determined as described in the text.

1960; Martin & Lindqvist, 1975). The pK_a for FITC appears to be lower by a few tenths of a unit (see below), so protonation will still occur in the range of interest.

FITC fluorescence intensity at 518 nm decreased with decreasing pH as expected from the pK_a and quantum yield data. As shown in Figure 3A, the ligands Ca^{2+}, phosphate, and vanadate had no effect, within experimental error, on the fluorescence intensity of the free probe. The dependency of FITC fluorescence on pH was reproduced with (solid line in Figure 3A)

$$I_{obsd} = I_D n_D + I_M n_M \quad (1)$$

where I_{obsd}, K_D, and I_M are the observed, dianion, and monoanion fluorescence intensities, respectively, and n_D and n_M and the fractions of dianion and monoanion present. I_D was taken as the relative fluorescence at pH 8.0, and $I_M = I_D(0.35/0.85)$. The values for n_D and n_M were determined at each pH from the Henderson–Hasselbalch equation. The best fit was obtained with $pK_a = 6.4$. The good agreement between the observed fluorescence intensity and that predicted from the known properties of fluorescein acidity and quantum yields demonstrates that no additional interactions are occurring. In this fit, there was only one variable parameter, the value for pK_a, and 6.4 is reasonable for fluorescein with an electron-withdrawing group attached (Leonardt et al., 1971).

Stern–Volmer slopes measured for I^- quenching of free FITC fluorescence in the presence of EGTA and plus Ca^{2+},

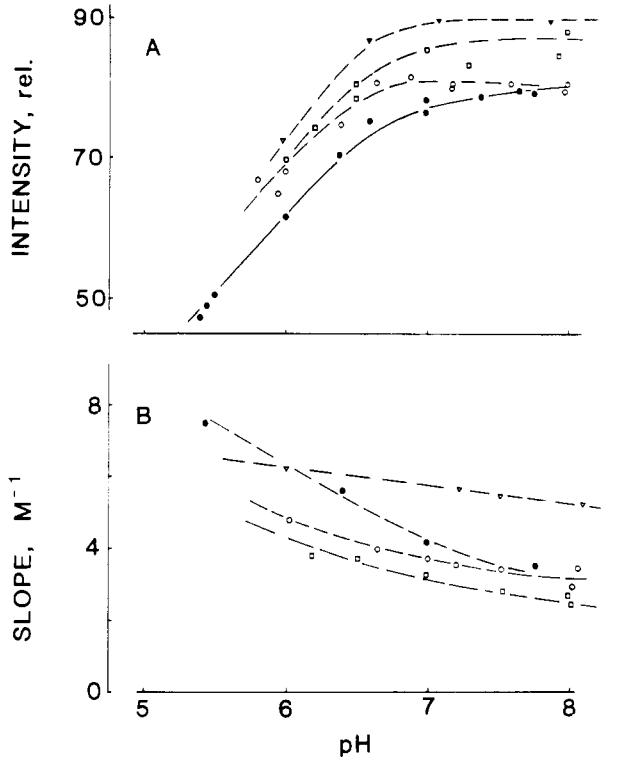

FIGURE 4: Iodide quenching of CaATPase-FITC fluorescence. (A) Intensities of SR CaATPase labeled with 1.1 nmol of FITC/mg of protein at 25 °C with 0.05 mg/mL SR protein, 100 mM KCl, 5 mM $MgCl_2$, 20 mM MOPs, 1 mM EGTA, and no addition (○), 1.1 mM $CaCl_2$ (●), 50 mM phosphate (□), or 25 μM vanadate (▽) at the pH indicated. (B) Stern–Volmer quenching slopes were obtained after addition of KI up to 38 mM. Solutions and symbols are as given for (A).

phosphate, or vanadate for pH 5–8, are shown in Figure 3B. As expected, the CaATPase ligands have no measurable effect on I^- quenching of the unattached probe. In Figure 3C, the apparent collisional quenching rate constant k_Q is plotted as a function of pH. The values for k_Q were obtained from $k_Q = K_Q/\tau$ (Vaughan & Weber, 1971; Lehrer, 1971), where the K_Q is from Figure 3B and τ is the effective average lifetime for the mixture of the dianion and anion estimated from the intensities in Figure 3A. The collisional constants (Figure 3C) are in the range expected for small negatively charged molecules being quenched by I^- (Lehrer, 1971). The increase in k_Q with decreasing pH and increasing amounts of monoanionic FITC is consistent with the observation that I^- quenching is enhanced about 2-fold when the fluorophore charge is increased by 1 (Lehrer, 1971).

Thus, the effects of pH, ligands, and I^- on the fluorescence intensity of free FITC in solution can be fully accounted for by known and reasonable values for its pK_a and quantum yields, by using the Stern–Volmer collisional quenching treatment and estimating the effective lifetime from the fluorescence intensity. These results will be used for comparison below to determine to what degree pH and ligand effects on CaATPase-FITC are due simply to direct effects on the FITC.

Ligand and pH Effects on CaATPase-FITC Fluorescence Intensity. The intensities of CaATPase-FITC at 518 nm were measured in the pH range 5–8 without ligands (EGTA alone) and with Ca^{2+}, phosphate, or vandate present (Figure 4A). In all cases, the intensity decreased with decreasing pH. The dependence of the intensity on pH could be fit with the treatment used for free FITC, above. The value for q_D was 0.60 (Highsmith & Murphy, 1984) and for q_m was 0.25,

Table I: Excited-State Lifetimes of CaATPase-FITC with Ligands Present

addition	lifetime (ns)[a]	intensity[b]
none	3.97 ± 0.06	80 ± 2
1.1 mM $CaCl_2$	4.09 ± 0.05	77 ± 2
25 μM vanadate	4.03 ± 0.07	90 ± 2
50 mM phosphate	3.99 ± 0.06	85 ± 2

[a] Measurements as described by Torgerson (1984) were made at 25 °C on 0.05 mg/mL SR protein with 2.1 nmol of FITC/mg of protein in 100 mM KCl, 20 mM MOPS, pH 7.0, 5 mM $MgCl_2$, 1.0 mM EGTA, plus additions as described above. [b] Steady-state measurements of relative fluorescence intensity. $CaCl_2$ and vanadate were added, and the intensity change was corrected for dilution. Phosphate data were obtained by adding equal volumes of CaATPase-FITC stock solutions to buffers ± phosphate. Six to ten measurements were made for each case.

obtained by assuming the monoanion/dianion ratio of quantum yields was the same as in the FITC case. With the pK_a for CaATPase-FITC as the variable parameter, the best fit obtained gave the solid line in Figure 4A and $pK_a = 5.8$.[2] Thus, the effects of pH on the fluorescence intensity of FITC-CaATPase are due to direct protonation of the probe.

Ligand binding, on the other hand, affected the fluorescence intensity but not the degree of protonation of the CaATPase-FITC. The excited-state lifetimes were constant (Table I), within experimental error, at pH 7.0, indicating that the ligand-induced effects were ground-state phenomena. It appears that the effects of ligand binding on the attached FITC are different from those due to changes in pH, which affect the FITC directly and change its quantum yield (Martin & Lindqvist, 1975). This result is compatible with a dynamic model of protein conformation that has a distribution of conformational substates for a given set of conditions. Ligand binding stabilizes some of the substrates more than others and changes the distribution. For the present case, some of the substrates are nonfluorescent, so the intensity can change without changes in the excited-state lifetime.

The ligand-induced changes in intensity (Table I) are in quantitative agreement with the results of others: Ca^{2+} causes a 4–5% decrease and vanadate causes a 10–12% increase in fluorescence intensity at pH 7.0 (Pick, 1982; Andersen et al., 1982; Highsmith, 1984). The effect of phosphate has not been reported. It increased the intensity, in percent, by 6.0 ± 1.5 over that of the EGTA case at pH 7.0.

Iodide Quenching of CaATPase-FITC. The slopes for Stern–Volmer quenching by I^- were determined for CaATPase-FITC in the pH range 6–8 for solutions containing EGTA alone and with Ca^{2+}, vanadate, or phophate added. In all cases, K_q increases with decreasing pH, indicating increases in the accessibility of the FITC (Figure 4B). The values are lower than those obtained for free FITC (Figure 3B). The values for K_q and values for excited-state lifetimes estimated from the intensities in the presence of Ca^{2+} were used to calculate the collisional rate constants for I^- quenching of the CaATPase-FITC fluorescence, as described above for FITC. As shown in Figure 5A, k_Q increases with decreasing pH for EGTA alone and for added phosphate or vanadate.

At all pH values, the vanadoenzyme complex has the attached FITC more accessible to I^- than the ligand-free enzyme, and the phosphoenzyme has FITC less accessible (Figure 5A). The k_Q value for CaATPase-FITC at pH 7.0 in the presence of phosphate is 37% of the value obtained for free FITC. This

[2] R. J. Coll and A. J. Murphy's unpublished observations of pH titrations of FITC-CaATPase absorbance at 490 nm give similar results.

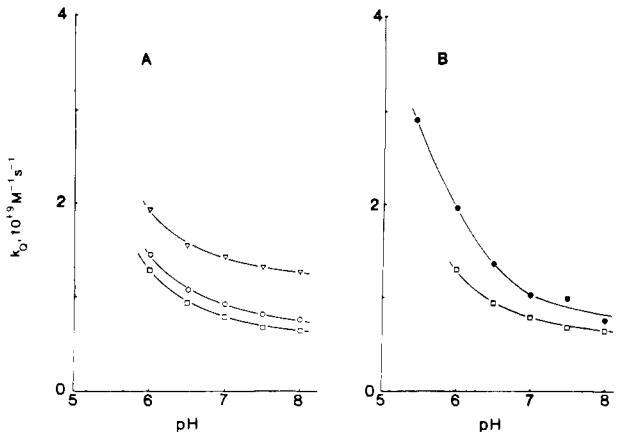

FIGURE 5: Collisional quenching constants for CaATPase-FITC. The collisional quenching constant k_Q was calculated from the fluorescence intensity and Stern–Volmer slopes in each case. The vesicular CaATPase-FITC was in solutions as described in Figure 4A, and the symbols have the same meaning. (A) The effects of vanadate and phosphate are compared to those for the ligand-free case. (B) The case with the high-affinity calcium binding sites filled is compared to that with the phosphoenzyme.

is consistent with the viscosity data (Figure 2), suggesting the FITC is in a protected environment. A much larger difference in the quenching of a different bound and free probe has been observed by Ando et al. (1982), who used acrylamide to quench a fluorescent derivative of ATP bound in the catalytic site of myosin. They found the quenching of the bound probe was decreased to 10% of that for the free probe, which was consistent with the observation that nucleotides can be completely occluded in the myosin ATP binding site (Wells & Yount, 1980).

The increases in the k_Q with decreasing pH (Figure 5A) are 2–4 times too large to be attributed completely to changes in the charge of the FITC moiety due to protonation (Figure 3A). A priori, there are only two likely mechanisms for this pH-dependent increase in k_Q. One is a reduction in steric hindrance, and the other is an increase in positive charge in the environment of the FITC. It is reasonable that increasing $[H^+]$ would increase the net positive charge on the surface of the CaATPase, including the area near the attached FITC. This is probably what is occurring; but changes in steric hindrance due to pH cannot be excluded.

The differences in k_Q induced by vanadate and phosphate, which are seen at all pH values, on the other hand, are more likely due to changes in steric hindrance. Phosphate and vanadate have similar charge and are thought to bind at the same location on the CaATPase, the phosphorylation site (Pick, 1981, 1982; Andersen et al., 1982; Highsmith et al., 1985; Csermely & Martonosi, 1985). The phosphorylation site may be near enough to the FITC to have a direct electrostatic effect. However, since vanadate causes an increase in the accessibility of the FITC to I^- and phosphate causes a decrease, when compared to the ligand-free enzyme (Figure 5A), it is implausible that the changes are due to the charge of the ligand. It is more likely that the ligand binding changes the enzyme conformation so that the FITC at the ATP binding site is exposed more or less to the bulk solvent and therefore available for I^- quenching by a collisional mechanism. The data in Figures 2 and 5A for CaATPase-FITC also are consistent with the FITC being located in a protected environment. It appears that vanadate and phosphate binding can modulate the degree of protection by changing the steric hindrance for I^- colliding with the attached FITC. An alternative is that phosphate and vanadate binding changes the protein surface charge near the FITC. This seems less likely than the steric mechanism, since the phosphate and vanadate curves are almost parallel over a range of two pH units (Figure 5A).

The effects of Ca^{2+} binding to CaATPase-FITC on I^- quenching are compared to the phosphoenzyme in Figure 5B. The notion that the Ca^{2+} effect is due to the charge of Ca^{2+} itself cannot be dismissed because Ca^{2+} binding causes an increase in k_Q (compared to the EGTA case, Figure 5A) at all pH and Ca^{2+} binding probably increases the net positive charge where it binds. Nonetheless, it seems unlikely that the Ca^{2+} effect on k_Q is due to a direct electrostatic effect. First, Ca^{2+} binding occurs at sites that appear to be at least 2 nm away (Highsmith & Murphy, 1984; Scott, 1984). This is too great a distance for a substantial electrostatic interaction in aqueous media containing 100 mM KCl. Second, the Ca^{2+} effect increases with decreasing pH. If Ca^{2+} were causing an increase in positive surface charge that increased k_Q, the effect would become smaller at lower pH with the expected increase in background protein surface positive. The opposite was observed, and a more likely explanation is that Ca^{2+} binding reduces a physical barrier to I^- quenching. Pyrene can be attached to sulfhydryl grouups that are thought to be near the ATP catalytic site, and their fluorescence quenching by the neutral molecule acrylamide is enhanced by Ca^{2+} binding (Kurtenbach & Verjovski-Almeida, 1985). This result is consistent with the steric interpretation of the iodide quenching data (Figure 5B).

A comparison that is of interest is between the Ca^{2+} and phosphate cases (Figure 5B). The data suggest that in going from E–P to ECa_2 the adenine binding subsite of the nucleotide binding site becomes 30% more accessible to the bulk solvent at pH 7; at pH 6, the increase is 48%. Interpreted as a change in steric hindrance, this corresponds to a substantial opening up of the nucleotide binding site due to Ca^{2+} binding. Interpreted in terms of an increase in positive charge, it would enhance the binding of the polyanion ATP. Regardless of the interpretation, the change in k_Q demonstrates that binding at the high-affinity Ca^{2+} binding sites causes structural changes at the ATP catalytic site. This interaction is complementary to the changes at the Ca^{2+} binding sites caused by ATP binding, observed by Stahl & Jencks (1984) and Murphy (1981).

The reverse reaction, ECa_2 to E–P, is also of interest. The data here suggest that phosphorylation of the CaATPase and calcium dissociation reduce the accessibility of the nucleotide binding site to the solvent. An effect of this kind has been suggested by de Meis and his co-workers (de Meis et al., 1980) on the basis of the effects of changes in solvent polarity on the kinetics of enzyme dephosphorylation.

In conclusion, FITC bound at the nucleotide catalytic site of the CaATPase behaves as if it were in an environment that is partially protected from the bulk solvent. A pocket or crevice on the enzyme surface would give such a result. The degree of exposure to the bulk solvent, as determined by iodide quenching, is sensitive to ligand binding. The results suggest that the ATP catalytic sites opens when Ca^{2+} binds to the high-affinity binding sites and closes when the enzyme is phosphorylated.

ACKNOWLEDGMENTS

It is a pleasure to thank Peggy Bloebaum for expert technical assistance, Dr. A. J. Murphy for making available some unpublished data on FITC protonation, Drs. Paul Torgerson and Thomas Burghardt for sharing the fluorescence lifetime instrument, and Dr. Andrzej Kasprzak for some useful discussions of the viscosity data.

Registry No. ATP, 56-65-5; FITC, 27072-45-3; I, 20461-54-5; ATPase, 9000-83-3; Ca, 7440-70-2; vanadate, 37353-31-4; phosphate, 14265-44-2.

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Direct NMR Evidence That Prolidase Is Specific for the Trans Isomer of Imidodipeptide Substrates†

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Received July 3, 1985

ABSTRACT: The in vitro hydrolysis by porcine kidney prolidase of the imidodipeptide L-alanyl-L-proline was monitored by using ^1H high-resolution NMR spectroscopy. The dipeptide exists as an equilibrium mixture of isomers with cis or trans conformation about the peptide bond. The ^{13}C and ^1H NMR spectra of the dipeptide displayed well-resolved resonances for each isomer. Inversion-transfer NMR spectroscopy, with a recently developed pulse sequence, was used with a range of temperatures to calculate the unitary rate constants for the exchange between isomers. A new analytical procedure was introduced for directly obtaining estimates of the unitary rate constants from inversion-transfer data. Arrhenius analysis yielded an activation energy for the isomerization of 87.0 ± 4.1 kJ mol^{-1}. ^1H NMR time courses of the prolidase-catalyzed hydrolysis of L-alanyl-L-proline showed a faster removal of the trans isomer as the [enzyme]/[substrate] ratio was increased. The transient-kinetic information coupled with the steady-state kinetic parameters of the enzyme was used to develop two possible models of the overall hydrolytic reaction. Numerical integration of the relevant differential equations using the experimentally determined rate constants gave simulated progress curves that enabled selection of one of the proposed schemes as being the most likely; this proposal entailed absolute specificity of prolidase for the trans isomer of L-alanyl-L-proline. Finally, on the basis of the present work, and information from the literature, we have proposed a new model of the active site of the enzyme.

Prolidase (proline dipeptidase; EC 3.4.13.9) is a highly specific exopeptidase, cleaving only those dipeptides with a C-terminal proline or hydroxyproline residue. The enzyme is anatomically ubiquitous in man and other animals, having been characterized from brain (Hui & Lajtha, 1978), erythrocytes (Endo et al., 1982), heart (Smith, 1948), intestinal mucosa (Rubino et al., 1969), kidney (Davis & Smith, 1957), leukocytes (Powell et al., 1974), liver (Norên et al., 1977),

†This work was supported by a grant from the Australian National Health and Medical Research Council. G.F.K. gratefully acknowledges the support of a Commonwealth Postgraduate Research Award.

0006-2960/86/0425-1054$01.50/0