Inactivation of H^+,K^+-ATPase by a K^+-Competitive Photoaffinity Inhibitor†

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Received October 5, 1987; Revised Manuscript Received January 12, 1988

ABSTRACT: A light-sensitive derivative, 2,3-dimethyl-8-[(4-azidophenyl)methoxy]imidazo[1,2-*a*]pyridine (DAZIP), of the drug 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine (SCH 28080) has been synthesized and shown to be a K^+-competitive inhibitor of gastric H^+,K^+-ATPase in the dark. The apparent dissociation constants calculated for DAZIP at pH 6.4 and 7.4 were 1.8 ± 0.2 and 4.7 ± 1.2 μM, respectively. Inhibition required binding of DAZIP to a luminal-facing site on the enzyme. Irradiation in the presence of DAZIP and 2 mM Mg^{2+} resulted in irreversible loss of ATPase activity that was more than 2-fold greater at pH 6.4 than at pH 7.4, showing the enhanced efficiency of covalent incorporation at the lower pH. Further photolyses were conducted at pH 6.4 in the presence of either 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA), ATP and CDTA, or MgATP. The specificity of light-dependent, covalent insertion of DAZIP for the site of reversible inhibition was shown both by protection against photoinactivation given by K^+ (the competing ligand) and by the observation that the amount of K^+-protectable photoinactivation approached a maximum limiting value as a function of DAZIP concentration. The effectiveness of K^+ in protecting against photoinactivation was 100-fold greater in the presence of ATP and CDTA than in the presence of either Mg^{2+} or CDTA and suggests the formation of a ternary complex of the apoenzyme with ATP and tightly bound K^+. The dissociation constant for DAZIP (2 μM) calculated from photolyses in the presence of MgATP without added K^+ agreed with the kinetic experiments and suggests that DAZIP inhibits turnover by binding to E·MgATP.

The gastric H^+,K^+-ATPase (Ganser & Forte, 1973; Sachs et al., 1976) is a member of a class of membrane-bound, ion-transporting ATPases that share a variety of functional and structural properties (Kyte, 1981a; Walderhaug et al., 1985). The α-polypeptide of Na^+,K^+-ATPase, in particular, shows a high degree of sequence homology (60%) when compared to H^+,K^+-ATPase (Shull & Lingrel, 1986). However, it has been found that the drug 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine (SCH 28080)[1] is an effective competitive inhibitor (K_i = 0.8 μM) with respect to K^+ in the case of H^+,K^+-ATPase but is at least 3 orders of magnitude less effective with Na^+,K^+-ATPase (Wallmark et al., 1987).

One of the interesting features of H^+,K^+-ATPase not shared with Na^+,K^+-ATPase is the requirement for extracytosolic K^+ binding in an environment of pH 1. The distinct differences in the apparent binding affinity for SCH 28080 may relate, therefore, to differences in structure at the external site for K^+. In order to begin to investigate this possibility, a photoaffinity derivative of SCH 28080, 2,3-dimethyl-8-[(4-azidophenyl)methoxy]imidazo[1,2-*a*]pyridine (DAZIP) has been prepared. This paper describes the interaction of DAZIP with H^+,K^+-ATPase. Both reversible inhibition in the dark and light-dependent, irreversible inactivation are observed, and each is antagonized by the presence of K^+. It is shown further that the inhibitor site is on the extracytoplasmic side of the membrane. Thus, DAZIP is a potential structural probe for a functionally significant luminal site on H^+,K^+-ATPase.

Experimental Procedures

Preparation of DAZIP. DAZIP (Figure 1) was synthesized by the Michigan State University Synthesis Laboratory according to the two-step procedure of Kaminski et al. (1985). In the first step, 2-amino-3-hydroxypyridine (0.09 mol) is refluxed for 20 h with 3-bromo-2-butanone (0.09 mol) in ethanol (80.0 mL) to form 8-hydroxy-2,3-dimethylimidazo-[1,2-*a*]pyridine in a condensation reaction [see method A of Kaminski et al. (1985)]. The bromide salt of the product, which precipitated from the reaction mixture, was collected by filtration, dissolved in water, and precipitated by neutralization with sodium bicarbonate (yield 20%). DAZIP is formed in the second step (method C, Kaminski et al., 1985). To the isolated condensation product (0.01 mol) dissolved in dimethylformamide (15.0 mL) was added sodium hydride (0.011 mol) in 50% mineral oil. After the mixture was stirred at 0 °C for 0.5 h, *p*-azidobenzyl bromide (0.011 mol) was added and the stirring continued for 12 h at 20 °C. The product was precipitated by addition of water, collected by filtration, dissolved in chloroform, dried, and recrystallized from ethyl acetate, mp 178–179 °C (yield 30%). The product was identified as DAZIP on the basis of the following spectral characteristics: IR (N_3 2120 cm^{-1}); NMR (singlets at 2.3, 2.4, and 5.22 ppm corresponding to CH_3, CH_3, and benzyl CH_2, respectively); mass spectrum (first peak of mass 265 daltons corresponding to the fragment of DAZIP given by loss of N_2).

Enzyme Preparation and Assays. Hog gastric H^+,K^+-ATPase microsomes were purified by zonal gradient centrifugation as previously reported (Rabon et al., 1985). Enzyme then was either diluted 10-fold with water, collected by centrifugation, and lyophilized or made up to 20% in sucrose and flash frozen. Unless otherwise specified, assays of lyophilized

† This research was supported by NIH Grant AM 17328-14 and the Los Angeles Veterans Administration Senior Medical Investigator Award.

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[1] Abbreviations: Pipes, 1,4-piperazinediethanesulfonic acid; CDTA, 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; DAZIP, 2,3-dimethyl-8-[(4-azidophenyl)methoxy]imidazo[1,2-*a*]pyridine; SCH 28080, 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DMSO, dimethyl sulfoxide; P_i, inorganic phosphate.

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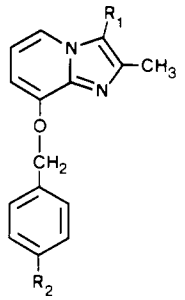
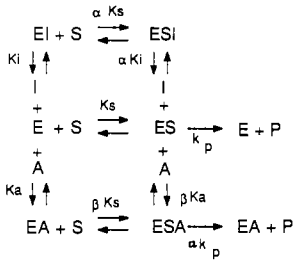


FIGURE 1: Structures of SCH 28080 and DAZIP. SCH 28080 (R_1 = CH_2CN, R_2 = H) and the photoaffinity derivative DAZIP (R_1 = CH_3, R_2 = N_3).

Scheme I[a]



[a] S is ATP, I is DAZIP, A is K^+, and K_s, K_i, and K_a are the dissociation constants for ATP, DAZIP, and K^+, respectively.

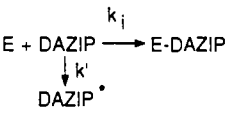
microsomes were in 40 mM Tris-HCl, 7.0 mM KCl, and 2.0 mM MgATP, pH 7.4, at 37 °C for 10–15 min. Activity was determined after acid quench by measuring inorganic phosphate in butyl acetate extracts of the assay solutions (Yoda & Hokin, 1970). Nigericin (5 μg/mL) and sucrose (20%) were included in assays of microsomes frozen in sucrose. By using this procedure to maintain isoosmotic conditions, the ionophore-independent ATPase was less than 10% of the stimulated activity. When photoinactivation of tight vesicles was measured, 0.1 M NH_4Cl was substituted for KCl. This eliminated the necessity of adding nigericin (Sachs, 1977) and provided close to the maximum observable activity without lyophilization. Photoinactivation of lyophilized enzyme by DAZIP was found to be identical whether assays were done with NH_4^+ or K^+, indicating that both methods measure the same population of H^+,K^+-ATPase. DAZIP was added to the assay mixtures after dilution of a 10 mM methanolic stock solution with DMSO. Therefore, all assays contained approximately 2% DMSO and a maximum of 0.5% methanol. Inhibition given by addition of these organic solvents without DAZIP was less than 5%.

Analysis of Reversible Inhibition. Reversible inhibition was analyzed according to a mechanism in which the binding of K^+ and DAZIP is mutually exclusive and K^+ is a nonessential activator [Scheme I; see Segel (1975)].

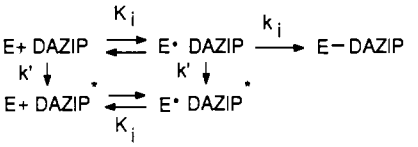
Velocity data were collected at a constant MgATP concentration while K^+ and DAZIP concentrations were varied. The values of αK_i and βK_A can be determined by rearranging the rate equation (Segel, 1975) into a form in which the reciprocal of the initial rate is expressed as a linear function of the inhibitor concentration. The resulting expression (Dixon equation) is closely approximated by eq 1 since an ATP concentration 50-fold greater than the K_s (40 μM) was used.

$$\frac{1}{v} = \frac{[I]}{V_{max}\alpha K_i[1 + (a[A]/\beta K_a)]} + \frac{1}{V_{max}}\left[\frac{[1 + (\beta K_a/[A])]}{[a + (\beta K_a/[A])]}\right] \quad (1)$$

Scheme II



Scheme III



By use of eq 1, values for βK_a were obtained from each intercept on the vertical axes of the Dixon plots, where

$$\beta K_a = \frac{[A](aV_{max} - v)}{v - V_{max}}$$

The parameters V_{max} and a were measured in separate assays.

Values for αK_i then were calculated from each βK_a and the corresponding intercepts on the horizontal axes of the Dixon plots, where

$$\alpha K_i = -[I]\frac{[a + (\beta K_a/[A])]}{[1 + (\beta K_a/[A])][1 + (a[A]/\beta K_a)]}$$

Photoinactivation Experiments. Enzyme was irradiated in 50 mM Pipes/Tris (buffer A) at either pH 6.4 or 7.4 with the additional substrate or ligands as indicated. Sucrose (20%) was included in photolyses of tight vesicles to inhibit rupture. DAZIP was added to photolysis mixtures as described for inhibition assays except that the dilution was 26-fold, giving 3.8% DMSO and a maximum of 0.6% methanol. These concentrations inhibited turnover by less than 15% and had no effect on activity when subsequently diluted 100-fold into the assay medium. Samples of 60–70 μL were placed in the wells of a small plastic titer plate (Costar, Cambridge, MA) and irradiated with a handheld ultraviolet lamp (Mineralight, Ultra-violet Products Inc., San Gabriel, CA) at 22 °C. The lamp was mounted on a stand at a distance of 4 cm from the solutions. Small aliquots (10 μL) of each sample were removed and added to the assay mixture (4 °C) before and after exposure to light for various lengths of time. All assays were conducted after completion of the photolysis.

Analysis of Photoinactivation Data. The fraction of enzyme covalently inactivated by light-dependent reaction with DAZIP was calculated for each time point by using eq 2 (Munson, 1981), where A_0 is defined as the activity in the absence of

$$\alpha_{EA}{}^t = 1 - \frac{A_{taz}A_0}{A_{0az}A_t} \quad (2)$$

DAZIP prior to irradiation, A_t is the activity in the absence of DAZIP after irradiation, A_{0az} is the activity in the presence of DAZIP prior to irradiation, and A_{taz} is the activity in the presence of DAZIP after irradiation.

Equation 2 corrects the raw inactivation data for both the small probability of light-dependent inactivation that occurs in the absence of inhibitor (evaluated in controls) and for any reversible inhibition relative to the control given by transfer of DAZIP to the assay mixture prior to irradiation.

The data were analyzed according to two possible mechanism of photoinactivation that have been presented previously (Kyte, 1981b), although both may occur simultaneously (see Schemes II and III). In these mechanisms k_i is defined as the pseudo-first-order rate constant for insertion of DAZIP into the inhibitor binding site; k' is the pseudo-first-order rate constant for alternative, nonproductive reactions; K_i is the

dissociation constant for DAZIP; DAZIP* is the unreactive product or products of the photolytic reaction of DAZIP that occurs even in the absence of enzyme; E-DAZIP is the modified enzyme that is no longer active; E·DAZIP is the reversible complex of the enzyme with unphotolyzed DAZIP; and E·DAZIP* is the reversible complex of the enzyme with the unreactive photolyzed derivatives of DAZIP.

For both of these mechanisms the level of photoinactivation (α_{EA}^{t}) is related to the time of exposure to the light by the expression

$$\ln (1 - \alpha_{EA}^{t}) = a(1 - e^{-k't}) \quad (3)$$

This equation was fitted numerically by first assuming a value for k' and then calculating the standard deviation in the parameter a given by each set of time course data. The value giving the minimum standard deviation was considered to represent the best fit.

As time of irradiation approaches infinity eq 3 becomes

$$\ln (1 - \alpha_{EA}^{\infty}) = a$$

The behavior of α_{EA}^{∞} as a function of the free concentration of DAZIP can be used to descriminate between Schemes II and III (Kyte, 1981b). The expression for Scheme II

$$\ln (1 - \alpha_{EA}^{\infty}) = -\frac{k_i}{k'}[\text{DAZIP}]_0 \quad (4)$$

shows that α_{EA}^{∞} must approach a value of 1.0 as the initial DAZIP concentration, $[\text{DAZIP}]_0$, approaches infinity. However, Scheme III predicts

$$\ln (1 - \alpha_{EA}^{\infty}) = -\frac{k_i}{k'}\left(\frac{[\text{DAZIP}]_F}{K_S + [\text{DAZIP}]_F}\right) \quad (5)$$

where $[\text{DAZIP}]_F$ is the free concentration of the inhibitor. In this case the ratio of the rate constants places an upper limit on α_{EA}^{∞}. If Scheme III may be considered a general mechanism, it is evident that no definite conclusion regarding the number of reversible binding sites present in solution should be based on the maximum attainable yields of photoinactivation (or covalent labeling) with photoaffinity reagents.

Equation 5 was fitted by assuming values for K_i and calculating the corresponding standard deviations in the ratio k_i/k' given by the collected set of α_{EA}^{∞} data. The K_i giving the minimum standard deviation was considered to represent the best fit.

Results and Discussion

Reversible Inhibition at pH 6.4 and 7.4. The inhibition of gastric acid secretion in dogs was studied previously for more than 80 different derivatives of the parent imidazopyridinium structure typified by SCH 28080 (Kaminski et al., 1985). The structure of DAZIP was designed from the structure–function relationships that emerged from this work. It was found in the present study that the essential aspect of inhibition of gastric H^+,K^+-ATPase by SCH 28080 in vitro, direct competition with K^+-activation, was also exhibited by DAZIP.

Inhibition of lyophilized H^+,K^+-ATPase by DAZIP was measured at both pH 6.4 and 7.4 since SCH 28080 is a more effective inhibitor at the lower pH (Wallmark et al., 1987). Plots of reciprocal initial velocity versus reciprocal K^+ concentration at different DAZIP concentrations (Figure 2) show sets of lines that intersect near the vertical axis for each pH. Only a minor change in apparent V_{max} was found, indicating that the binding of DAZIP and K^+ is mutually exclusive. At high concentrations, 60 μM, a change in V_{max} was found, presumably indicating additional sites of inhibition.

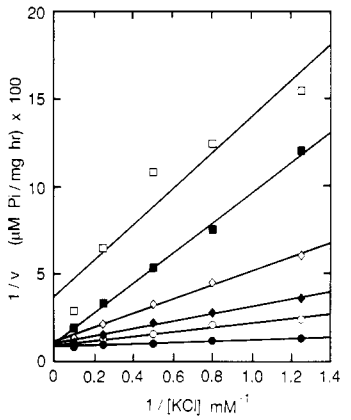


FIGURE 2: Inhibition of H^+,K^+-ATPase in the dark. Initial velocities of K^+-stimulated ATPase were measured at 37 °C in solutions of H^+,K^+-ATPase suspended in buffer A with 2 mM $MgCl_2$, 2 mM Na_2ATP, pH 6.4, and the indicated concentrations of KCl. DAZIP concentrations were 0 (●), 5 (○), 10 (◆), 20 (◇), 40 (■), and 60 (□) μM. Lines were drawn by linear regression analysis.

Inhibition was greater at pH 6.4 than at 7.4 for any given concentration of DAZIP. In order to extract the apparent dissociation constants for DAZIP (αK_i) and K^+ (βK_a) for these initial velocity data, Scheme I was used as a model. In this mechanism, K^+ is a nonessential activator that binds to the enzyme in direct competition with DAZIP. The factor of activation (parameter a in Scheme I) was determined in separate assays by measuring initial velocities at various ATP concentrations, either in the absence of K^+ or in the presence of saturating K^+ (2.5 mM). When the reciprocal plots (velocity versus ATP) were extrapolated to infinite substrate concentration, it was found that the maximum initial rate in the presence of K^+ was 11.8-fold greater than in its absence.

Values for βK_a and αK_i were calculated from eq 1 and the intercepts on the vertical and horizontal axes of the Dixon plots, respectively, which were linear as predicted by Scheme I. The analyses yielded αK_i of 1.8 ± 0.2 and 4.7 ± 1.2 μM, at pH 6.4 and 7.4, respectively, and βK_a of 0.45 ± 0.12 mM at pH 6.4 and 0.34 ± 0.18 mM at pH 7.4. Thus, in the presence of saturating MgATP, the inhibitor binds to gastric H^+,K^+-ATPase with more than 2-fold higher apparent affinity at pH 6.4 than at pH 7.4. With eq 1 and the values determined for αK_i and βK_a, it can be calculated that the concentration of K^+ required to give half-maximal velocity at pH 6.4 in the presence of 10 μM DAZIP is 2.8 mM.

Inhibition of the nigericin-stimulated ATPase in vesicles "tight" to K^+ was measured at pH 6.4. These microsomes are made up to 20% in sucrose after zonal gradient purification and immediately flash-frozen at low temperature, thus maintaining minimal K^+ permeability. Their activity is stimulated 10-fold or more by nigericin to the same maximal value as seen with washed and lyophilized vesicles.

As a control, a portion of the same tight vesicle preparation was diluted 10-fold into water and then lyophilized and resuspended to the original volume in order to retain the same zonal buffer and protein components as the tight vesicle preparation. As expected, this treatment resulted in a maximum of 1.3-fold stimulation by nigericin in 10 mM KCl, while the original vesicles were 12.3-fold stimulated. Thus, the control vesicles had been made permeable to K^+ under the assay conditions.

Inhibition of both the lyophilized control and the tight vesicles by DAZIP was strictly competitive with K^+. Analysis of the control velocity data gave nearly the same values of αK_i

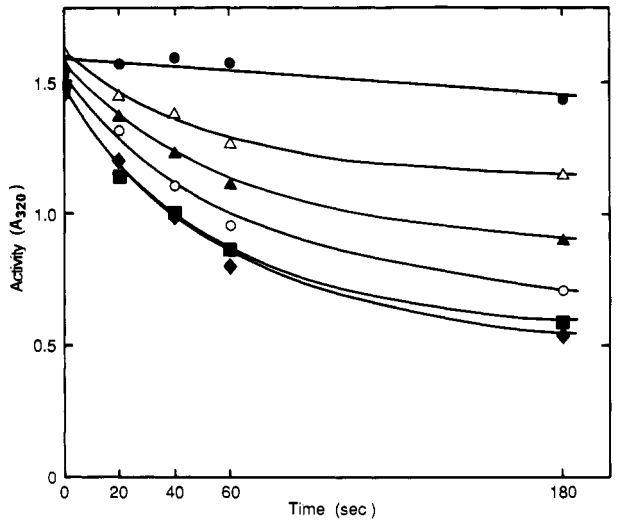


FIGURE 3: Time course of photoinactivation. Solutions (60 μL) of resuspended, lyophilized H^+,K^+-ATPase (0.8 mg/mL) in buffer A, pH 6.4, 2 mM $MgCl_2$, and various concentrations of DAZIP were irradiated simultaneously at 22 °C in small, open-titer wells. At the times indicated, 10-μL aliquots were removed from each sample and diluted into 0.89 mL of standard assay mix without ATP at 4 °C. After completion of the photolyses, the activities were measured (A_{320}). Curves represent the best fit to eq 3. Concentrations of DAZIP were (from top to bottom) 0, 5, 10, 20, 40, and 60 μM.

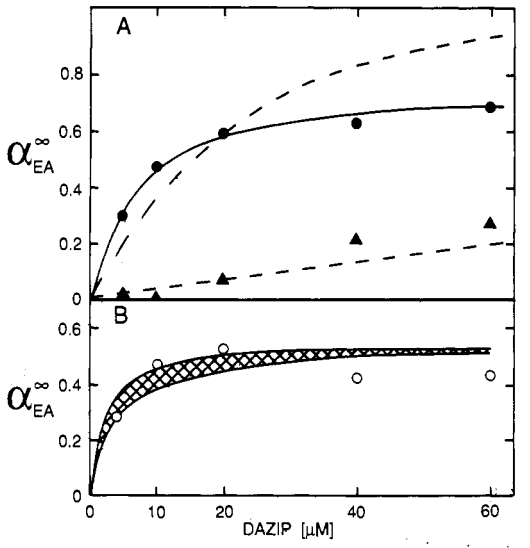


FIGURE 4: Photoinactivation in the presence of Mg^{2+}. The fraction of photoinactivated H^+,K^+-ATPase (α_{EA}^{∞}) given by the indicated concentrations of DAZIP was determined from photolysis time courses by the best fits to eq 3. (A) Photolyses were conducted without KCl (●) or in the presence of 50 mM KCl (▲) at pH 6.4. The best fits to eq 5 (Scheme III) are represented by solid lines and to eq 4 (Scheme II) by dashed lines. (B) The differences in α_{EA}^{∞} given by addition of KCl are plotted versus DAZIP concentration. The shaded region is defined by curves drawn according to eq 5 with K_i values of either 6 (lower boundary) or 3 μM (upper boundary).

for DAZIP (2.4 μM) and βK_a for K^+ (0.53 mM) as found in the case of washed, lyophilized membranes. Thus, the minor amounts of contaminating proteins and buffer components (e.g., Ficoll) present in the tight vesicle preparation had no effect on inhibition. However, different results were obtained for nigericin-stimulated ATPase in tight vesicles, although nigericin alone had no effect on DAZIP inhibition of lyophilized vesicles. The βK_a values for K^+ were 40, 20, and 6 mM for the solution concentrations of 1, 2, and 10 mM KCl, respectively, while the αK_i values calculated from each βK_a were 78, 39, and 37 μM, respectively. Considering that more than 90% of the H^+,K^+-ATPase in tight vesicles is oriented with the cytoplasmic domain of the protein on the outside (Sachs et al., 1980), it was concluded that inhibition of H^+,K^+-ATPase with an apparent inhibition constant of 2 μM DAZIP does not occur on the cytoplasmic domain of the enzyme but on the luminal face, as for SCH 28080.

Photoinactivation in the Presence of Mg^{2+}. In order to examine the irreversible, light-dependent inactivation given by DAZIP, lyophilized H^+,K^+-ATPase was irradiated at pH 6.4 and 7.4 in the presence of 2 mM $MgCl_2$ and various concentrations of the inhibitor. In addition, other sets of photolyses were conducted under the same conditions but including 50 mM KCl to protect the enzyme against covalent modification at the inhibitor binding site. The inhibition experiments in the dark suggested that a concentration of 50 mM K^+ would be sufficient to prevent any measurable, reversible binding of DAZIP. A relatively high concentration of protein was used (0.8 mg/mL) in order to maximize the concentration of binding sites for DAZIP and to allow for a large dilution (100-fold) of the enzyme mixtures into assay medium. Thus, only inactivation produced by covalent modification was detected during the course of the photolysis experiments. A range of DAZIP concentrations was used that, on the basis of reversible kinetics, would give saturation of the inhibitor site. Small portions of enzyme mixtures were irradiated and the time courses of light-dependent inactivation measured. Under all conditions, DAZIP produced photoinactivation that approached an apparent maximum over a period of time similar to that found to give destruction of the azido group. Controls irradiated in the absence of DAZIP always showed less than 10% photoinactivation. A typical time course experiment is shown in Figure 3. The data were analyzed according to the two alternative mechanisms for covalent photoinactivation above (Kyte, 1981b). The analyses can provide sufficient evidence to eliminate an undesired mechanism (Scheme II) and provide support for one in which reversible binding precedes covalent modification (Scheme III). In the case of Scheme II, inactivation results from a simple bimolecular reaction of the protein with the light-activated inhibitor. This mechanism may reflect "nonspecific photoinactivation" since no complex of the inhibitor with the enzyme is required prior to covalent attachment. In contrast, Scheme III specifies that covalent photoinactivation can occur only when the inhibitor is bound in a reversible complex with the enzyme.

Analysis of the time course data first required the use of eq 2 to calculate α_{EA}^{t}, the fraction of enzyme inactivated by covalent reaction with the light-activated compound at each time point. The time courses under each set of conditions then could be fitted to eq 3, which expresses α_{EA}^{t} as a function of the length of time the sample was exposed to the light. As an example, the best fits to the data collected at pH 6.4 in the absence of K^+ were calculated and then used to draw the curves in Figure 3. It can be seen that this procedure yielded curves that closely matched the observed inactivation.

Equation 3 describes the kinetics of photoinactivation for either Scheme II or III (each contains different unknown constants within the parameter a), and therefore the analyses to this point do not allow elimination of either mechanism. However, eq 3 approaches $-\ln(1 - \alpha_{EA}^{\infty}) = a$ as time (t) approaches infinity. The value of the parameter a determined from the best fits to eq 3 at each DAZIP concentration allows for the direct calculation of α_{EA}^{∞}, the fraction of the enzyme that would be inactivated at infinite time of irradiation. It is the change in the value of α_{EA}^{∞} as a function of DAZIP concentration that may allow for the elimination of Scheme II.

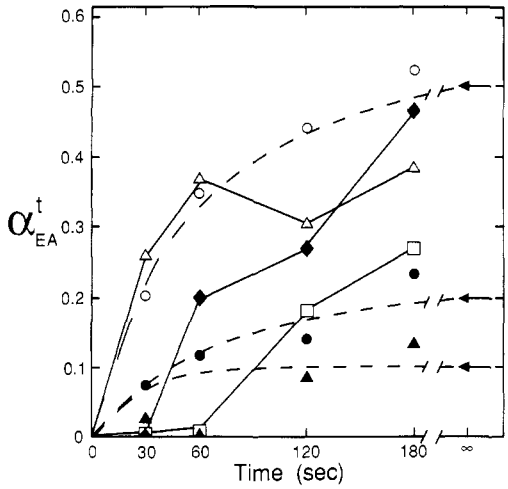


FIGURE 5: Effect of ATP on photoinactivation with 10 μM DAZIP. The fraction of enzyme inactivated, $\alpha_{EA}{}^t$, is shown as a function of time. Irradiation was with 10 μM DAZIP in buffer A, 0.5 mM CDTA, pH 6.4 (●), and either 0.1 mM NaAMP (▲) or 0.10 (△), 0.2 (○), 0.5 (◆), or 1.0 mM Na_2ATP (□). Arrows indicate the values of $\alpha_{EA}{}^\infty$, and the dotted lines represent the best fits to eq 3 given by photolyses in the presence of 0.2 mM Na_2ATP (upper), 0.5 mM CDTA (middle), and 0.1 mM NaAMP (lower).

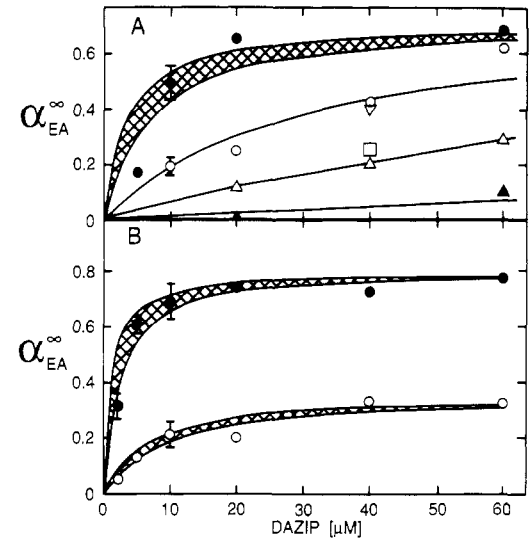


FIGURE 6: Photoinactivation in the presence of CDTA, ATP, or MgATP. (A) Lyophilized enzyme was irradiated with the indicated concentrations of DAZIP in buffer A with 0.5 mM CDTA (○), and either 50 mM KCl (△), 10 mM KCl (□), 1.0 mM KCl (▽), 0.2 mM Na_2ATP (●), or 0.2 mM Na_2ATP and 10 mM KCl (▲). (B) Enzyme irradiated in the same buffer with DAZIP and either 2.0 mM $MgCl_2$ and 0.2 mM Na_2ATP (●), or 2.0 mM $MgCl_2$, 0.2 mM Na_2ATP, and 50 mM KCl (○). Photoinactivation ($\alpha_{EA}{}^\infty$) was calculated from the best fits to eq 3. Error bars indicate the standard deviation in measurements at selected concentrations of DAZIP (n ranged from 2 to 9). The curve for photoinactivation in the presence of CDTA (A) was drawn by the best fit to eq 5. Other curves forming the boundaries of the shaded areas were drawn by using eq 5 assuming a maximum $\alpha_{EA}{}^\infty$ of 0.7 and K_i values of 5 or 10 μM for photolyses in the presence of ATP (A), 0.8 and K_i values of 2 or 4 μM in the presence of MgATP (B), or 0.35 and K_i values of 7 or 10 μM in the presence of MgATP and 50 mM KCl (B).

The calculated $\alpha_{EA}{}^\infty$ values for photolyses at pH 6.4 are plotted as a function of DAZIP concentration in Figure 4. The data obtained in the presence of 50 mM K^+ were fit reasonably to eq 4 (dotted lines, Figure 4). Since it was shown that reversible binding of DAZIP is nearly abolished in the presence of 50 mM K^+ (see Figure 2) it was assumed that photoinactivation under these conditions is predominantly the result of covalent modification at sites other than the specific DAZIP binding site.

An identical set of photoinactivation experiments at pH 7.4 showed that the maximum K^+-protectable activity was less than half that seen at pH 6.4. Furthermore, the levels of photoinactivation ($\alpha_{EA}{}^\infty$) observed in the presence of K^+ were two-fold higher at all DAZIP concentrations examined up to 60 μM, suggesting an increased reactivity of the arylnitrene for certain residues on the surface of the protein at pH 7.4.

The values of $\alpha_{EA}{}^\infty$ calculated from photolyses at pH 6.4 in the absence of K^+ were fit both to eq 4 and eq 5 (Scheme III). Scheme II (nonspecific photoinactivation) could be eliminated, while a reasonable fit to eq 5 was found. An apparent dissociation constant of 13 μM for the reversible binding of DAZIP was calculated by using the data to find the best fit to eq 5. However, when the apparently nonspecific photoinactivation ($\alpha_{EA}{}^\infty$ in the presence of K^+) was subtracted point by point from $\alpha_{EA}{}^\infty$ obtained in the absence of K^+, a new set of values was generated that logically represent inactivation resulting only from the covalent attachment of DAZIP at the inhibitor binding site. These new values were fitted to eq 5 (Figure 4B, shaded areas) and an arbitrary range of reasonable values for K_i, 3–6 μM, was found. A 2-fold increase in the apparent dissociation constant was observed when photoinactivation data obtained at pH 7.4 were analyzed by this procedure. Thus, the dissociation constants obtained from the photoinactivation experiments agreed (within a factor of 2) with those calculated earlier from reversible inhibition. These results suggest that K^+-protectable photoinactivation observed in the presence of Mg^{2+} is given by covalent attachment of DAZIP at the inhibitory site to which it binds reversibly in the dark.

Photoinactivation in the Presence of CDTA or ATP. It was reported previously that SCH 28080 exhibits uncompetitive inhibition of gastric H^+,K^+-ATPase with respect to ATP (Wallmark et al., 1987). This suggested that DAZIP inhibits while bound in a complex of the enzyme with the substrate (Scheme I). In order to examine the possible effect of ATP on photoinactivation under nonturnover conditions, time courses of photoinactivation were measured at pH 6.4 in 0.5 mM CDTA with 10 μM DAZIP and several concentrations of either ATP, ADP, ADP and P_i, or AMP (Figure 5). No phosphorylation of the enzyme occurs at this concentration of CDTA (Wallmark & Mardh, 1979). Concentration-dependent enhancement of photoinactivation was given by ATP but not by AMP (0.05, 0.1, or 0.5 mM), ADP (0.4 mM), or ADP (0.4 mM) and P_i (1.0 mM). Each of these conditions gave $\alpha_{EA}{}^\infty$ less than 0.1. Maximum enhancement to an $\alpha_{EA}{}^\infty$ of 0.5 occurred in 0.2 mM ATP, while higher concentrations (1 mM) appeared to give a filter effect that delayed the time course of photoinactivation. Addition of CDTA without ATP reduced the photoinactivation 2.5-fold to an $\alpha_{EA}{}^\infty$ of 0.2. Thus, similar levels of $\alpha_{EA}{}^\infty$ were given by 10 μM DAZIP in the presence of either ATP and CDTA or 2 mM Mg^{2+}, but 0.5 mM CDTA gave sharply lowered photoinactivation.

It was of interest to determine if the presence of ATP (instead of Mg^{2+}) would increase the apparent affinity of DAZIP calculated from photoinactivation experiments. Time courses of photoinactivation for several concentrations of DAZIP were measured in the presence of CDTA (0.5 mM) or CDTA and ATP (0.2 mM) at pH 6.4 (Figure 6A). Nonspecific photoinactivation again was determined by the inclusion of K^+ in parallel sets of photolyses. Complete protection in the presence of ATP was given by 10 mM K^+ except at 60 μM DAZIP, where protection was 85%. Therefore, eq 5 was fit directly to the values of $\alpha_{EA}{}^\infty$ obtained in the absence of K^+, and a K_i of 7 μM was calculated.

Lower values for α_{EA}^{∞} were found at all DAZIP concentrations in the presence of CDTA, and K^+ did not protect as effectively. It appeared that the protective effect of 50 mM K^+ was nearly at a maximum under these conditions. This was shown by the observation that only a minor increase in protection against 40 μM DAZIP in CDTA occurred when the concentration of K^+ was raised from 10 to 50 mM (Figure 6A). It was clear from this result that the apparent high affinity for K^+ in the presence of ATP and CDTA was not owing simply to the absence of free Mg^{2+} but was due to an ATP effect on apoenzyme. The apparent binding affinity for DAZIP also was lower in the presence of CDTA. After subtraction of the photoinactivation measured in the presence of 50 mM K^+, the best fit to eq 5 yielded a K_i of 18 μM, while a direct fit without subtraction gave a K_i of 43 μM. Thus, the apparent K_i for DAZIP, as determined from the photoinactivation data at pH 6.4, is similar in the presence of either Mg^{2+} or ATP but apparently increases in the presence of CDTA. These data provide further evidence for a conformational effect upon binding of Mg^{2+} (Helmich-DeJong et al., 1986) or ATP (Wallmark & Mardh, 1979) to the apoenzyme.

The possibility that the apparent saturation of α_{EA}^{∞} at values close to 0.5 results from access of DAZIP to only half of the inhibitory sites (with half of sites binding giving full reversible inhibition) was examined in double-photolysis experiments. Two photoinactivation time courses of 3-min duration were measured after successive additions of saturating DAZIP (22 μM) in the presence of either ATP or Mg^{2+}. In each case, the fraction of photoinactivated enzyme calculated from eq 2 was 0.45 ± 0.01 after the first photolysis and 0.52 ± 0.02 of the remaining activity after the second. The overall fractional photoinactivation (without extrapolation to infinite time of exposure) was 0.76 with ATP present and 0.74 with Mg^{2+} present. Thus, these experiments provided no evidence for half-of-sites reactivity with respect to DAZIP binding.

Photoinactivation in the Presence of MgATP. It was of interest to measure the effect of MgATP on the levels of photoinactivation given by DAZIP since SCH 28080 progressively decreased the levels of phosphorylated enzyme (by up to 90%) in the same concentration range that inhibits turnover (Wallmark et al., 1987). Lyophilized H^+,K^+-ATPase at pH 6.4 was mixed with DAZIP and ATP (0.2 mM). After the addition of either Mg^{2+} (2 mM) or Mg^{2+} and K^+ (50 mM), photolyses were performed immediately, and α_{EA}^{∞} was calculated from the time courses of photoinactivation. The values of α_{EA}^{∞} as a function of DAZIP concentration are shown in Figure 6B. The data could not be fit reasonably to eq 4, and a simple bimolecular mode of photoinactivation was ruled out. The best fits to eq 5 gave a K_i of close to 2 μM whether or not the α_{EA}^{∞} values found in the presence of K^+ were subtracted as background. Thus, the binding affinity as determined from photoinactivation experiments is at least 2-fold greater in the presence of MgATP when compared to either ATP without Mg^{2+} or Mg^{2+} alone and agrees most closely with the K_i obtained from reversible inhibition. This result suggests only a slight difference in the conformation of the site under these different circumstances. It is proposed that during turnover DAZIP binds to E·MgATP from the luminal side of the membrane to inhibit phosphorylation.

Surprisingly, photoinactivation in the presence of MgATP and 50 mM K^+ appeared to approach a limiting α_{EA}^{∞} at high DAZIP concentrations and, therefore, could not be fit reasonably to eq 4. A possible explanation for this observation would be that the arylnitrene generated by adsorption of light

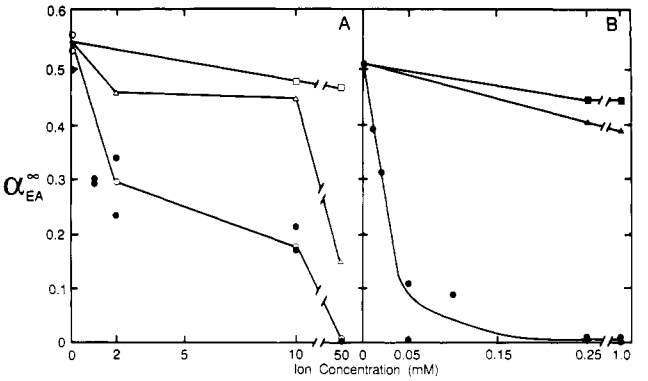


FIGURE 7: Effect of K^+ on photoinactivation. [A (open symbols)] Resuspended, lyophilized H^+,K^+-ATPase was irradiated in buffer A with 10 μM DAZIP, 2 mM $MgCl_2$ and the indicated concentrations of either KCl (○), NaCl (△), or TMACl (□). (●) A tight vesicle preparation of H^+,K^+-ATPase was irradiated in buffer A, 0.5 mM CDTA, 0.2 mM Na_2ATP, and 20% sucrose with 10 μM DAZIP and the indicated concentrations of KCl. (B) Resuspended, lyophilized H^+,K^+-ATPase was irradiated with 10 μM DAZIP in buffer A, 0.2 mM Na_2ATP, 0.5 mM CDTA, and the indicated concentrations of KCl (●), NaCl (▲), or KCl with 2 mM $MgCl_2$ (■). In each case α_{EA}^{∞} was calculated from time course data and the best fits to eq 3.

may have a longer lifetime in solution than when bound. In this case the sites may be covalently modified during turnover even though only a small fraction of them are occupied by the inhibitor at any time. This situation (pseudophotoaffinity labeling) could exist in the presence of a concentration of K^+ that prevents any measurable, reversible inhibition by DAZIP.

Protection against Photoinactivation Given by K^+. The specificity of the photoinactivation reaction for the inhibitor binding site was tested further by examining the relative effectiveness of different monovalent ions in protecting lyophilized enzyme against photoinactivation. Values of α_{EA}^{∞} were calculated from time courses of photoinactivation at pH 6.4 in the presence of 10 μM DAZIP, 2 mM Mg^{2+}, and various concentrations of K^+, Na^+, or TMA^+ (Figure 7A, open symbols). The order of effectiveness found for protection ($K^+ > Na^+ > TMA^+$) parallels the activation of H^+,K^+-ATPase and provides evidence for the specificity of photoinactivation. Furthermore, it was found that the concentration of K^+ (3–4 mM), required to reduce the α_{EA}^{∞} by half, was close to that required for half-maximal velocity (2.8 mM) under turnover conditions at pH 6.4 in the presence of 10 μM DAZIP.

The surprisingly high level of protection given by 10 mM K^+ in the presence of ATP led to a more detailed examination of this effect. Lyophilized gastric H^+,K^+-ATPase was photoinactivated with DAZIP (10 μM) in the presence of ATP (0.2 mM) and CDTA (0.5 mM). Protection against photoinactivation given by different concentrations of K^+ and Na^+ was determined (Figure 7B). In some of the photolyses, Mg^{2+} (2 mM) was added along with K^+. The concentration of K^+ required to give a 2-fold reduction in the α_{EA}^{∞} was 20–30 μM and was specific for K^+ since Na^+ gave little protection even at 1 mM. When Mg^{2+} was added along with K^+ to the photolysis mixture, the high-affinity protection was abolished, and as a consequence, 1 mM K^+ gave only an 11% reduction in α_{EA}^{∞}. It was concluded that although the complexes E·Mg^{2+} and E·ATP show little difference in their dissociation constants for the inhibitor, the latter appears to bind K^+ with 100-fold higher apparent affinity. This is an unexpected result since, in the case of Na^+,K^+-ATPase, it appears that a K^+-"occluded" form of the enzyme releases tightly bound K^+ to the cytosol upon the binding of ATP (Karlish & Yates, 1978). One observation supporting this conclusion is that K^+–K^+ exchange catalyzed by Na^+,K^+-ATPase of red cells shows a requirement

for either ATP or its $\beta\gamma$-imido or methylene analogues (Glynn et al., 1975). In contrast, ATP does not stimulate, but at concentrations of 0.1 mM or greater, inhibits Rb^+–Rb^+ exchange mediated either by native H^+,K^+-ATPase or by the H^+,K^+-ATPase reconstituted asymmetrically into proteoliposomes (Rabon et al., 1985). A shift to high affinity for K^+ upon binding of ATP in the absence of Mg^{2+} would provide one explanation for this result. Nevertheless, it is not clear that tightly bound K^+ in the presence of ATP is not also a property of Na^+,K^+-ATPase under some conditions. It was shown (Askari et al., 1980) that cross-linking of the α-polypeptide of canine Na^+,K^+-ATPase with Cu^{2+} and *o*-phenanthroline was abolished only when both ATP and K^+ were present. Apoenzyme (EDTA treated) was used, but the final pH of the cross-linking mixture after addition of the nucleotide was not specified. In approximate aggreement with the present experiments, however, the half-maximal inhibitory effect occurred at 0.2 mM ATP and 30 μM K^+. The cross-linking reaction required several minutes to come to completion, and it follows that a rapidly transient complex of the enzyme with ATP and K^+ (Karlish & Yates, 1978) would not be expected to inhibit cross-linking.

Experiments were done to determine if the high-affinity protection by K^+ in the presence of ATP occurs by binding to the cytosolic or luminal (external) side of H^+,K^+-ATPase. Tight vesicles were irradiated with DAZIP and ATP in the absence or presence of nigericin and a range of K^+ concentrations. Photoinactivation in the presence of 0.2 mM ATP and 10 μM DAZIP was the same as the ATP-enhanced values (α_{EA}^{∞} of 0.5) previously seen with lyophilized vesicles, indicating DAZIP could enter tight vesicles. However, in contrast to results obtained with lyophilized vesicles, addition of 1.0 mM K^+ failed to give complete protection. Instead, the decrease in α_{EA}^{∞} with increasing K^+ was similar to the protection curve seen for photoinactivation of lyophilized enzyme with 10 μM DAZIP in the presence of Mg^{2+} alone and the concentration of K^+ required to reduce α_{EA}^{∞} 2-fold was 2–5 mM (Figure 7A). When nigericin was added to allow penetration of the tight vesicles by K^+, complete protection against photoinactivation again was given by 1 mM KCl. Therefore, K^+ is required on the interior (luminal-facing side) of the vesicles in order to bind with high affinity in the presence of ATP but apparently also can bind with lower affinity from the cytosolic side. Since the concentration of K^+ required for half-maximal turnover in the presence of 10 μM DAZIP was close to that giving low-affinity protection, it appears that reversible competition with DAZIP in the presence of MgATP occurs by binding of K^+ from the cytosolic face of the enzyme. This would be consistent with the lack of any measurable rapid effect by SCH 28080 on the rate of K^+-stimulated dephosphorylation of gastric H^+,K^+-ATPase, which has been shown to require luminal K^+ (Wallmark et al., 1987).

It is interesting to note that occupation of the high-affinity luminal site by K^+ afforded almost complete protection against photoinactivation by DAZIP even at the highest concentrations of the inhibitor examined. However, under conditions of lower affinity protection (i.e., K^+ with Mg^{2+}, MgATP, or CDTA) substantial levels of photoinactivation were still observed, even though the kinetics of inhibition in the dark suggested that no DAZIP should be reversibly bound. An explanation for these observations would be that a greater number of nonspecific sites for covalent modification by DAZIP are shielded or buried when K^+ binds with ATP than under the other conditions tested.

In summary, DAZIP has been shown to satisfy the requirements of a photoaffinity probe. Photoinactivation at pH 6.4 is prevented specifically by the natural ligand K^+ and shows behavior that is consistent with the formation of a reversible complex prior to light-dependent, covalent inactivation. These criteria are met by DAZIP for several distinct conformational states of gastric H^+,K^+-ATPase and have provided additional evidence for ATP and Mg^{2+} forms of the enzyme. Light-dependent labeling with radioactive DAZIP may identify a region of primary sequence within the extracellular domain of the polypeptide and detect changes in conformation at this site associated with different ligand conditions.

Registry No. DAZIP, 114095-26-0; ATPase, 9000-83-3; 2-amino-3-hydroxypyridine, 16867-03-1; 3-chloro-2-butanone, 4091-39-8; 8-hydroxy-2,3-dimethylimidazo[1,2-*a*]pyridine, 114095-27-1; 8-hydroxy-2,3-dimethylimidazo[1,2-*a*]pyridine hydrobromide, 114095-28-2; *p*-azidobenzyl bromide, 74489-49-9; potassium, 7440-09-7.

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