Eds.) Vol. 10, pp 449-464, Raven Press, New York, NY.
Heidelberger, C. (1975) *Annu. Rev. Biochem. 47*, 79-121.
Herskovits, T. T. (1967) *Methods Enzymol. 11*, 748-775.
Huberman, E., Sachs, L., Yang, S. K., & Gelboin, H. V. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 607-611.
Ibanez, V., Geacintov, N. E., Gagliano, A. G., Brandimarte, S., & Harvey, R. G. (1980) *J. Am. Chem. Soc. 102*, 5661-5666.
King, H. W., Osborne, M. R., Beland, F. A., Harvey, R. G., & Brookes, P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2679-2681.
Koreeda, M., Moore, P. D., Yagi, H., Yeh, H. J., & Jerina, D. M. (1976) *J. Am. Chem. Soc. 98*, 6720-6722.
Li, H. J., & Crothers, D. M. (1969) *J. Mol. Biol. 39*, 461-477.
MacLeod, M. C., & Selkirk, J. K. (1982) *Carcinogenesis (London) 3*, 287-292.
MacLeod, M. C., Smith, B., & McClay, J. (1987) *J. Biol. Chem. 262*, 1081-1087.
Manning, G. S. (1978) *Q. Rev. Biophys. 11*, 179-246.
Marcus, R. A. (1965) *J. Chem. Phys. 43*, 1261-1274.
McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469-489.
Meehan, T., Straub, K., & Calvin, M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1437-1441.
Meehan, T., Gamper, H., & Becker, J. F. (1982) *J. Biol. Chem. 257*, 10479-10485.
Nelson, H. P., Jr., & DeVoe, H. (1984) *Biopolymers 23*, 897-911.
Paulius, D. E., Prakash, A. S., Harvey, R. G., Abramovich, M., & LeBreton, P. R. (1986) *Polynuclear Aromatic Hydrocarbons: Chemistry, Characterization and Carcinogenesis* (Cooke, M., & Dennis, A. J., Eds.) pp 745-754, Battelle Press, Columbus, OH.
Philpott, M. R. (1970) *J. Chem. Phys. 53*, 968-981.
Ramstein, J., Ehrenberg, M., & Rigler, R. (1980) *Biochemistry 19*, 3938-3948.
Record, M. T., Jr., Anderson, C. F., & Lohman, T. M. (1978) *Q. Rev. Biophys. 11*, 103-178.
Sims, P., Grover, P. L., Swaisland, A., Pal, K., & Hewer, A. (1974) *Nature (London) 252*, 326-328.
Straub, K. M., Meehan, T., Burlingame, A. L., & Calvin, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5285-5289.
Tunis-Schneider, M. J. B., & Maestre, M. F. (1971) *J. Mol. Biol. 52*, 521-541.
Wei, C. S.-J., Desai, S. M., Harvey, R. G., & Weiss, S. B. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5936-5940.
Weinstein, I. B., Jeffrey, A. M., Jennette, K. W., Blobstein, S. H., Harvey, R. G., Harris, C., Autrup, H., Kasai, H., & Nakanishi, K. (1976) *Science (Washington, D.C.) 193*, 592-595.
Wells, R. D., Larson, J. E., Grant, R. C., Shortle, B. E., & Cantor, C. R. (1970) *J. Mol. Biol. 54*, 465-497.
Wilson, W. D., & Lopp, I. G. (1979) *Biopolymers 18*, 3025-3041.
Wolfe, A., Shimer, G. H., Jr., & Meehan, T. (1987) *Biochemistry 26*, 6392-6396.
Yang, N.-C. C., Hrinyo, T. P., Petrich, J. W., & Yang, D.-D. H. (1983) *Biochem. Biophys. Res. Commun. 114*, 8-13.
Yang, S. K., McCourt, D. W., Gelboin, H. V., Miller, J. R., & Roller, P. P. (1977) *J. Am. Chem. Soc. 99*, 5124-5130.

Inhibition and Derivatization of the Renal Na,K-ATPase by Dihydro-4,4′-diisothiocyanatostilbene-2,2′-disulfonate†

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ABSTRACT: Treatment of purified renal Na,K-ATPase with dihydro-4,4′-diisothiocyanatostilbene-2,2′-disulfonate (H_2DIDS) produces both reversible and irreversible inhibition of the enzyme activity. The reversible inhibition is unaffected by the presence of saturating concentrations of the sodium pump ligands Na^+, K^+, Mg^{2+}, and ATP, while the inactivation is prevented by either ATP or K^+. The kinetics of protection against inactivation indicate that K^+ binds to two sites on the enzyme with very different affinities. Na^+ ions with high affinity facilitate the inactivation by H_2DIDS and prevent the protective effect of K^+ ions. The H_2DIDS-inactivated enzyme no longer exhibits a high-affinity nucleotide binding site, and the covalent binding of fluorescein isothiocyanate is also greatly reduced, but phosphorylation by P_i is unaffected. The kinetics of inactivation by H_2DIDS were first order with respect to time and H_2DIDS concentration. The enzyme is completely inactivated by the covalent binding of one H_2DIDS molecule at pH 9 per enzyme phosphorylation site, or two H_2DIDS molecules at pH 7.2. H_2DIDS binds exclusively to the α-subunit of the Na,K-ATPase, locking the enzyme in an E_2-like conformation. The profile of radioactivity, following trypsinolysis and SDS-PAGE, showed H_2DIDS attachment to a 52-kDa fragment which also contains the ATP binding site. These results suggest that H_2DIDS treatment modifies a specific conformationally sensitive amino acid residue on the α-subunit of the Na,K-ATPase, resulting in the loss of nucleotide binding and enzymatic activity.

The Na,K-ATPase (EC 3.6.1.3) is the plasma membrane enzyme that couples the transmembrane transport of Na^+ and K^+ ions to the hydrolysis of ATP (Glynn & Karlish, 1975). ATP hydrolysis is catalyzed through a reaction cycle involving conformational changes of the protein with Na^+-dependent phosphorylation from ATP and subsequent K^+-dependent breakdown of the phosphoenzyme. In recent years significant progress has been made in linking the biochemical transformations catalyzed by the enzyme to the transmembrane ion movements (Kaplan, 1983, 1985). However, most of the

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important structural features are still unknown, and data on the effects of chemical modification of the enzyme should provide important information relating structural and mechanistic properties of the enzyme.

P_i[1] and ATP are known to phosphorylate an aspartyl residue on the enzyme (Walderhauch et al., 1985), and there is some information regarding the possible participation of other amino acid residues at the locus of binding of the physiological ligands (DePont et al., 1977; Cantley et al., 1978; Karlish, 1980; Patzelt-Wenczler & Schoner, 1981; Shull et al., 1985; Kawakami et al., 1985; Schneider et al., 1985). Since the substrate contains negatively charged phosphate groups, the existence of some positively charged recognition sites on the enzyme for ATP seems likely. The modification of two different lysyl residues on the α-subunit with FITC (Farley et al., 1984) or FSBA (Ohta et al., 1986) has previously been shown to inactivate the enzyme. In the present study, the effect of a negatively charged amino-reactive reagent, H_2DIDS, on the Na,K-ATPase has been examined. We describe observations which show that H_2DIDS produces both inhibition and inactivation[2] of the enzyme with a single amino acid residue modified in the α-subunit of the Na,K-ATPase. The reactivity of this site toward H_2DIDS can be regulated by physiological ligands of the sodium pump. Preliminary reports of some of these findings have been previously presented (Pedemonte & Kaplan, 1988, 1986a).

Materials and Methods

Materials. FITC,[1] ATP, pNPP, BSA, AMP, UTP, and ADP were from Sigma Chemical Co. H_2DIDS and [^{3}H]-H_2DIDS (sp act. 202 mCi/mmol) were from Research Development Corp., Toronto, Canada, in some early experiments. The majority of the experiments employed [^{3}H]H_2DIDS that was obtained as a generous gift from Dr. M. L. Jennings (Galveston). [^{32}P]P_i and [^{3}H]ADP were from Amersham. Acrylamide, bis(acrylamide), and 2-mercaptoethanol were from Bio-Rad Labs. SITS was from Molecular Probes and DNDS from Pfaltz and Bauer, Inc. BIDS was synthesized as indicated by Rao et al. (1979). All other chemicals were of the highest quality available.

Enzyme Isolation and Assays. Na,K-ATPase was purified from dog kidneys according to Jorgensen (1974) with the modifications of Liang and Winter (1976). The enzyme was greater than 95% pure as judged from SDS–polyacrylamide gel electrophoresis (PAGE) (see Figure 6). The standard assay medium for Na,K-ATPase activity was (mM) EGTA, 0.5; NaCl, 130; KCl, 20; $MgCl_2$, 3; ATP, 3; imidazole, 50; pH 7.2 (20 °C); and about 0.8 μg/mL enzyme protein. Unless specified, it also contained 0.3 mg/mL BSA. The suspension was incubated at 37 °C for 15 min and the P_i released determined as reported by Brotherus et al. (1981). The Na,K-ATPase activity was the difference between the ATP hydrolysis measured in the absence and presence of 5×10^{-4} M ouabain. The enzyme used in these studies had a specific activity of 15–20 μmol of P_i released mg^{-1} min^{-1}, a negligible ouabain-insensitive ATP hydrolysis, and maximal phosphorylation by P_i at 37 °C in the presence of ouabain of about 2.7 nmol/mg. Protein was determined by the method of Lowry et al. (1951) using BSA as standard. The *p*-nitrophenylphosphatase (pNPPase) activity was measured at 37 °C in an assay medium containing (mM) EGTA, 0.5; $MgCl_2$, 5; KCl, 20; Tris–*p*-nitrophenyl phosphate (pNPP), 3; and imidazole, 50; pH 7.2 (20 °C). Unless specified, it also contained 0.3 mg/mL BSA. The pNPPase activity was the difference between the pNPP hydrolysis measured in the absence and presence of 5×10^{-4} M ouabain.

Phosphorylation by [^{32}P]P_i was performed as described by Askari and Huang (1982). ADP binding was obtained as indicated by Robinson (1980). Treatment of the enzyme (1 mg/mL) with FITC was performed at room temperature in a medium containing (mM) Tris-HCl, pH 9 (37 °C), 50; EDTA, 1; NaCl, 80; and FITC, 0.03 (Hegyvary & Jorgensen, 1981). After 45 min, the suspension was diluted with 2-mercaptoethanol and BSA (100 mM and 1 mg/mL final concentrations, respectively) and centrifuged at 436000*g* for 5 min. The pellet was washed with 25 mM imidazole and 1 mM EDTA, pH 7.5 (37 °C) (buffer C), once more and resuspended in the same buffer.

SDS–PAGE was carried out by using the buffer system of Laemmli (1970) on a linear gradient gel (8–14%). After the gels were stained with Coomassie blue and destained, the gels were photographed and cut in 5-mm slices which were solubilized with 0.5 mL of 30% H_2O_2, and the radioactivity was determined. Fluorescent bands produced by FITC-labeled enzyme were visualized in unstained gels by illumination in a darkroom with a long-wavelength UV lamp and photographed by using TRI-X film and a yellow filter.

Trypsin treatment of the enzyme was performed as indicated by Jorgensen (1975). Additional experimental conditions are detailed in the figure legends. In the figures, "remaining activity" represents the ratio v_i/v_0, where v_0 and v_i are the enzyme activity before and after inhibition or inactivation by H_2DIDS, respectively.

Treatment with H_2DIDS. Concentrated stock solutions of H_2DIDS or [^{3}H]H_2DIDS (2–5 mM) in H_2O were stored in small aliquots at −20 °C until use. The UV absorption spectrum of H_2DIDS in 50 mM phosphate buffer (pH 7) was periodically measured, and the concentration was calculated from the absorption at 285 nm by using a value of 3.45×10^4 for the molar extinction coefficient (Research Development Corp. bulletin). Inactivation of the enzyme by H_2DIDS (or labeling with [^{3}H]H_2DIDS) was performed at 37 °C by incubating 1 mg/mL of enzyme for 30 min in a medium containing 50 mM Tris-HCl, 1 mM EDTA, pH 9 or 7.2 (37 °C), and 12 μM H_2DIDS. The reaction was stopped by a 3-fold dilution of the suspension with 400 mM mercaptoethanol and 16 mg/mL BSA, followed by centrifugation at 436000*g* for 5 min. The pellet was washed three times with a solution of BSA (4 mg/mL) in buffer C and once with buffer C alone. The pellet was finally resuspended in buffer C; protein, radioactivity content (if [^{3}H]H_2DIDS was used), and Na,K-ATPase and pNPPase activities were then determined. This preparation is referred to as "H_2DIDS–enzyme". Another way of measuring inactivation by H_2DIDS was also employed. After "preincubation" of the enzyme with H_2DIDS in 30 μL of a medium containing 50 mM Tris-HCl, 1 mM EDTA, pH 9 or 7.2 (37 °C), and variable H_2DIDS concentrations, the reaction was halted with 20 μL of 125 mM 2-mercaptoethanol and 0.5 mg/mL BSA. The suspension was then diluted 12-fold with the assay medium containing all the enzyme ligands, including 0.3 mg/mL BSA. Both methods gave the same result.

[1] Abbreviations: FITC, fluorescein 5′-isothiocyanate (isomer 1); SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; BIDS, 4-benzamido-4′-isothiocyanatostilbene-2,2′-disulfonate; P_i, inorganic phosphate; H_2DIDS, 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

[2] "Inactivation" always refers to irreversible inhibition.

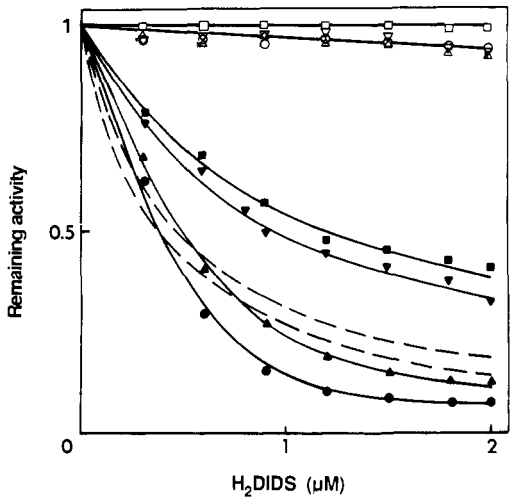


FIGURE 1: Reversible inhibition of Na,K-ATPase and pNPPase activities by H_2DIDS. Na,K-ATPase (■, □, ▼, ▽) and pNPPase (△, ▲, ○, ●) were measured in the absence (○, ●, ▽, ▼) and presence (△, ▲, □, ■) of 50 mM 2-mercaptoethanol and in the absence (■, ▲, ▼, ●) and presence (□, △, ▽, ○) of 0.3 mg/mL BSA. H_2DIDS at the indicated concentrations was added to the assay medium containing the enzyme (0.5 μg) and its ligands. The suspension was incubated for 15 min at 37 °C, and the hydrolysis of ATP was then determined. *Continuous lines* through the Na,K-ATPase data were drawn according to the equation $v_i/v_0 = K_i/(K_i + I)$ (Segel, 1975), where v_0 and v_i are the enzyme activity before and after addition, respectively, of H_2DIDS (I). K_i is the dissociation constant of H_2DIDS. Lines were fitted by using values for K_i of 1.0 and 1.2 in the absence and presence of 2-mercaptoethanol, respectively. Lines through the pNPPase activity data were drawn by eye. *Dashed lines* represent the values predicted by the above equation for the pNPPase activity by using as K_i the I_{50} obtained from the experimental data, e.g., 0.40 and 0.44 in the absence and presence of 2-mercaptoethanol, respectively. The composition of the assay media is detailed under Materials and Methods. BSA was not present during assays except when its effect on the reversible inhibition was determined.

Unless specified, *reversible inhibition* was measured in the presence of saturating concentrations of all the enzyme ligands required for activity, while *inactivation* was determined in their absence.

RESULTS

Characteristics of Reversible Inhibition and Inactivation. H_2DIDS inhibits Na,K-ATPase and pNPPase activities when added directly to the enzyme assay media in the presence of saturating concentrations of the enzyme ligands (Figure 1). Full activity is recovered by washing the enzyme (by centrifugation and resuspension) free of H_2DIDS, indicating that the H_2DIDS inhibition is *reversible*. The fact that saturating concentrations of the different enzyme ligands did not affect the reversible inhibition suggests that H_2DIDS behaves as a simple noncompetitive inhibitor (Segel, 1975; Dixon & Webb, 1979). An equation derived for this type of inhibition (Segel, 1975) fit the Na,K-ATPase activity data very well but not that of the pNPPase activity (Figure 1). H_2DIDS concentrations necessary to reduce the initial enzyme activity to 50% (I_{50}) were 1 μM and 0.4 μM for the Na,K-ATPase and the pNPPase activities, respectively. For a simple noncompetitive inhibitor the $I_{50} = K_i$ (Segel, 1975). The presence of 50 mM 2-mercaptoethanol, which reacts with the isothiocyanate groups of H_2DIDS, only slightly reduced the reversible inhibition. The presence of 0.3 mg/mL BSA almost totally protected the Na,K-ATPase and pNPPase activities against the inhibition by H_2DIDS (Figure 1). This was a very useful way of preventing the interaction between H_2DIDS and the enzyme and of stopping the progress of the inactivation (see below). The

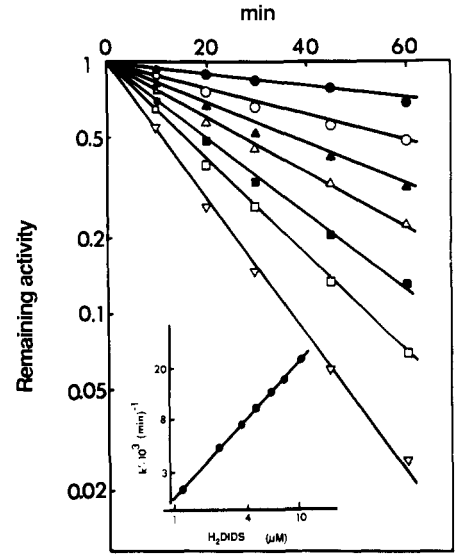


FIGURE 2: Time course of inactivation of Na,K-ATPase by H_2DIDS. The enzyme (0.033 mg/mL) was incubated at 37 °C in the presence of the following concentrations of H_2DIDS (μM): 1.2 (●), 2.4 (○), 3.6 (▲), 4.8 (△), 6.4 (■), 8 (□), and 11 (▽). At the indicated times, aliquots of 30 μL were withdrawn and put into tubes containing 20 μL of 125 mM 2-mercaptoethanol and 0.75 mg/mL BSA to stop the reaction. Na,K-ATPase activity was assayed as indicated under Materials and Methods in the presence of 0.3 mg/mL BSA. Continuous lines are the representation of the equation $\log v_i/v_0 = -k_1 I^n t/2.3K_i$, where v_0 and v_i are the enzyme activity before and after inactivation, respectively, n is the number of H_2DIDS molecules that produce the inactivation, K_i is the dissociation constant of H_2DIDS, k_1 is the first-order rate constant of inactivation, and t is the time of treatment. The values used were 0.0045 min^{-1} μM^{-1} for k_1/K_i and 1.1 for "n". Inset: Logarithmic plot of the apparent first-order rate constant of inactivation ($k' = k_1 I^n/K_i$) versus H_2DIDS concentration.

greater inhibition of the pNPPase compared with that of the Na,K-ATPase activity was not due to protection by Na^+ or ATP since 100 mM NaCl, 100 mM choline hydrochloride, or varying ATP concentrations did not affect the reversible inhibition of the phosphatase activity by H_2DIDS (data not shown). K^+ and Mg^{2+} were also discounted as being responsible for the differential inhibition as they were present at more or less the same concentration in both the Na,K-ATPase and pNPPase assay media (see Materials and Methods).

The effects of pH on the reversible inhibition by H_2DIDS were examined. The control enzyme activities were (μmol of P_i mg^{-1} min^{-1}) 10.6, 19.2, 19.0, and 13.5 at pH 6.5, 7.2, 7.9, and 8.4, respectively. This variation of Na,K-ATPase activity with pH is very similar to that previously described by Skou (1979). When the pH of the Na,K-ATPase assay medium was increased, the inhibition due to H_2DIDS was reduced. The residual Na,K-ATPase activity was 27%, 42%, 65%, and 76% at pH 6.5, 7.2, 7.9, and 8.4, respectively, at 37 °C in the presence of 2 μM H_2DIDS. Therefore, it seems likely that a residue on the protein that is involved in the reversible inhibition is titrated in this pH range. The reversible inhibition of the pNPPase activity by H_2DIDS shows much less sensitivity to pH (data not shown).

When the enzyme was preincubated with H_2DIDS in the presence of 50 mM Tris-HCl and 1 mM EDTA, pH 9 (37 °C), in the *absence* of the enzyme ligands and then assayed in the presence of saturating levels of the ligands, inactivation of the Na,K-ATPase was observed (Figure 2). The activity could not be recovered by extensive washing of the enzyme with buffer C and either BSA or 2-mercaptoethanol. However, addition to the preincubation medium of a large molar excess

of either 2-mercaptoethanol or BSA preserved the enzyme activity. The I_{50} value for H_2DIDS after 60-min incubation at 37 °C was 1.4 μM. Other analogues of H_2DIDS, BIDS and SITS, gave values of 1.9 μM and 2.9 μM, respectively, under the same experimental conditions (Pedemonte & Kaplan, 1988). The reaction between these inhibitors and the enzyme probably first involves reversible interactions, between the negatively charged sulfonic acid groups of H_2DIDS, SITS, or BIDS and some positively charged groups on the protein, and then a covalent reaction ensues between the –NCS group(s) of the reagents and side-chain amino residues of the enzyme. The reaction can be described by

$$E \overset{I}{\underset{K_i}{\rightleftharpoons}} (EI) \xrightarrow{k_1} E_i$$

where I is the inhibitor which reversibly interacts with the free enzyme (E) with a dissociation constant K_i. The complex (EI) is converted in a unimolecular reaction to the inactivated form (E_i) with a rate constant k_1.

The data in Figure 2 were fit by using an equation (legend to Figure 2) previously derived for this kind of reaction (Pedemonte & Kaplan, 1986b). Analysis of the inactivation data by using this equation implies that covalent binding of H_2DIDS to the enzyme proceeds in a single exponential phase with a rate constant $k' = k_1 I^n / K_i$. A logarithmic plot of the remaining Na,K-ATPase activity (v_i/v_0) versus time of treatment with H_2DIDS gave a linear relationship at all H_2DIDS concentrations used (Figure 2). The apparent first-order rate constants of inactivation are replotted in the inset of Figure 2, which shows a linear relationship between log k' and log [H_2DIDS]. The closeness of the slope to unity ("n" = 1.1) is consistent with the assumption that the binding of one molecule of H_2DIDS per enzyme molecule is sufficient to produce the inactivation.

The idea that the formation of a reversible complex between H_2DIDS and the protein, which is responsible for the reversible inhibition, is a precursor to the inactivation reaction was supported by experiments using DNDS. DNDS is a stilbenedisulfonic acid where the isothiocyanate groups of H_2DIDS are replaced by nitro groups so that covalent bond formation with the protein after reversible binding could not occur. Like H_2DIDS, DNDS behaves as a simple noncompetitive inhibitor of the Na,K-ATPase activity (Pedemonte & Kaplan, 1988). DNDS inhibited with an I_{50} of about 1.75 mM, which is more than 3 orders of magnitude lower affinity than that observed for H_2DIDS under the same conditions. As expected from the structural similarities, DNDS and H_2DIDS behaved as mutually *exclusive* inhibitors as if both compounds compete for the same binding site(s) on the enzyme (Pedemonte & Kaplan, 1988). This was also supported by the observation that when present during the treatment of the enzyme with H_2DIDS (in the absence of ligands), DNDS prevented the inactivation of the Na,K-ATPase in a concentration-dependent fashion.

Monovalent Cation Effects. Since the enzyme is only reversibly inhibited by H_2DIDS when all its physiological ligands are present (Figure 1) and it is inactivated when they are absent (Figure 2), all or some of the enzyme ligands must protect against the irreversible modification. K^+, when present during the preincubation of the enzyme with H_2DIDS, preserved the enzyme activity (Figure 3). This effect was saturable, and at higher K^+ concentrations than were necessary for the maximal protection, the effect was reduced. The concentration dependence of the first phase of K^+ protection was hyperbolic with a half-maximal effect ($K_{0.5}$) of 80 μM

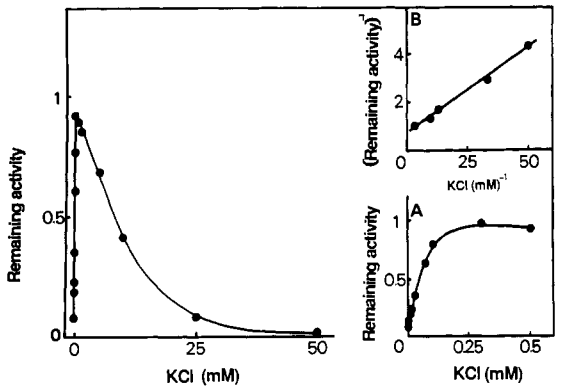


FIGURE 3: Effect of K^+ on the inactivation of Na,K-ATPase by H_2DIDS. The enzyme (0.033 mg/mL) was incubated for 30 min at 37 °C in the presence of 50 mM Tris-HCl, 1 mM EDTA–Tris, pH 9 (37 °C), 12 μM H_2DIDS, and different KCl concentrations. The inactivation reaction was halted by addition of 50 mM 2-mercaptoethanol and 0.3 mg/mL BSA. Na,K-ATPase activity was assayed as indicated under Materials and Methods. Inset A: The protective effect of low KCl concentrations shown on an expanded scale. Inset B: Double-reciprocal plot of the data shown in inset A.

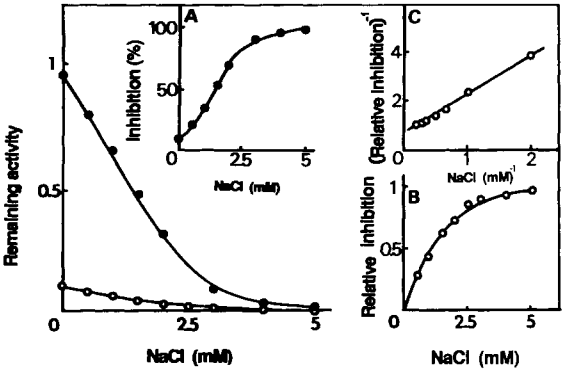


FIGURE 4: Effect of Na^+ on the H_2DIDS inactivation of Na,K-ATPase. The enzyme (0.033 mg/mL) was incubated for 30 min at 37 °C in the presence of 50 mM Tris-HCl, 1 mM EDTA–Tris, pH 9 (37 °C), 12 μM H_2DIDS, different NaCl concentrations, and plus (●) or minus (○) 0.3 mM KCl. The reaction of H_2DIDS was stopped by addition of 50 mM 2-mercaptoethanol and 0.3 mg/mL BSA. The Na,K-ATPase was assayed as indicated under Materials and Methods. Inset A: The inhibition produced by the presence of Na^+ (when counteracting the protective effect of K^+) is replotted to show the "sigmoidal" dependency. Inset B: Stimulation by Na^+ of the inactivation of the enzyme by H_2DIDS. This inhibition is replotted to show the "hyperbolic" dependency. Inset C: Double-reciprocal plot of the data of inset B.

(Figure 3A,B). The second phase of the K^+ action shows a $K_{0.5}$ of about 9 mM (Figure 3).

Na^+ produced a facilitation of the H_2DIDS inactivation of the Na,K-ATPase with a $K_{0.5}$ of about 2 mM (Figure 4). The relative inhibition versus [Na^+] followed a hyperbolic relation (Figure 4B,C), suggesting that the facilitating effect of Na^+ is accomplished by only one Na^+ ion interacting with the enzyme. Addition of low concentrations of Na^+ to a reaction medium containing 0.3 mM KCl along with the enzyme and H_2DIDS counteracted the protective effect of K^+ (Figure 4). The Na^+ effect follows a sigmoid relation (Figure 4A), Na^+ being half-maximally effective at about 2 mM.

Nucleotide Binding and Protection. Mg^{2+} (1 mM) had little effect on the inactivation of the Na,K-ATPase by H_2DIDS (Figure 5). ATP and ADP protected against H_2DIDS inactivation (in the absence of Mg^{2+}) with a $K_{0.5}$ of about 90 μM (Figure 5). No evidence was obtained (from thin-layer chromatography or UV absorption spectra) of a direct interaction between ATP, which has an amino group on the

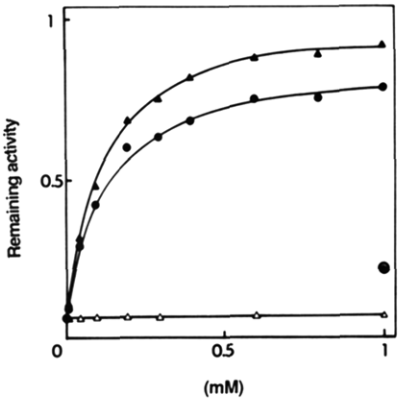


FIGURE 5: Effect of nucleotide phosphates on the inactivation of Na,K-ATPase by H_2DIDS. The enzyme (0.033 mg/mL) was incubated for 30 min at 37 °C in the presence of 50 mM Tris-HCl, 1 mM EDTA–Tris, pH 9 (37 °C), and different concentrations of ATP (▲), ADP (●), UTP (△), or AMP (△). The reaction was stopped by addition to the medium (30 μL) of 20 μL of 2-mercaptoethanol (125 mM) and BSA (0.75 mg/mL). The Na,K-ATPase activity was assayed as indicated under Materials and Methods. The effect of 1 mM free Mg^{2+} on the inactivation is also shown (●).

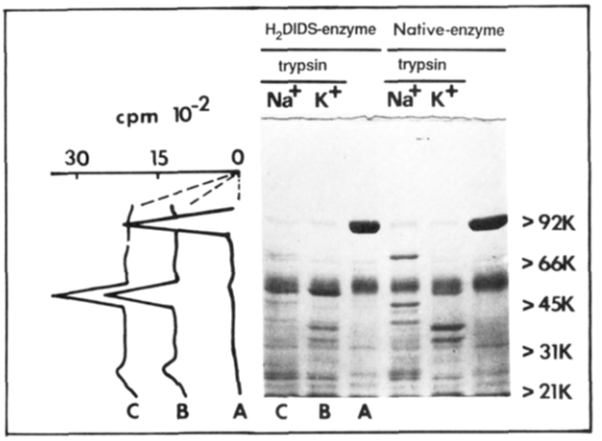


FIGURE 6: Polyacrylamide gel electrophoresis of trypsin-treated H_2DIDS–enzyme. H_2DIDS–enzyme was prepared, treated with trypsin in the presence of either Na^+ plus ADP (lane Na^+) or K^+ (lane K^+), and subjected to SDS–PAGE as indicated under Materials and Methods. The positions of molecular weight markers, run on the same gel, are indicated. Na,K-ATPase not treated with H_2DIDS (native enzyme) and H_2DIDS treated (H_2DIDS–enzyme) are indicated. Third lanes are enzyme not treated with trypsin. The gel whose picture is shown in the figure was cut, dissolved in H_2O_2, and counted for radioactivity as indicated under Materials and Methods. The picture shows the radioactivity profile of enzyme before (A) and after treatment with trypsin in the presence of either Na^+ plus ADP (C) or K^+ (B).

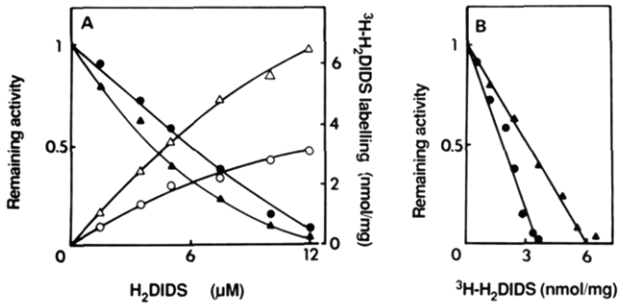


FIGURE 7: Correlation between [^{3}H]H_2DIDS binding and inactivation of Na,K-ATPase activity. (A) The enzyme (1 mg/mL) was incubated at 37 °C for 60 min in the presence of 50 mM Tris-HCl, 1 mM EDTA–Tris, pH 9 (37 °C) (●, ○) or 7.2 (▲, △), and different concentrations of [^{3}H]H_2DIDS. After extensive washing, Na,K-ATPase activity (●, ▲) and radioactivity content (○, △) were determined. (B) Linear relationship between inactivation of the enzyme and H_2DIDS binding at pH 7.2 (▲) and 9 (●). Data were taken from panel A.

adenine ring, and H_2DIDS. This was also supported by the lack of protection against H_2DIDS inactivation afforded by AMP, which binds poorly to the ATP site on the enzyme. The high-affinity binding of [^{3}H]ADP, as an ATP analogue (Askari & Huang, 1982), to the H_2DIDS–enzyme was reduced compared to control enzyme. ADP binding was 0.12 ± 0.04 and 2.34 ± 0.05 nmol/mg to H_2DIDS–enzyme and native enzyme, respectively. The 95% decrease in nucleotide binding correlated well with the reduction in Na,K-ATPase activity. Experiments with FITC also suggest an alteration in the ATP binding region. FITC inactivates the Na,K-ATPase, and ATP competes with this effect with a high affinity (Karlish, 1980; Hegyvary & Jorgensen, 1981; Sen et al, 1981). The binding of FITC to the α-subunit of the enzyme was greatly reduced by previous H_2DIDS treatment (data not shown). DNDS, which protects the enzyme against H_2DIDS inactivation and binding, also protected against FITC binding.

Even though the ATP binding region on the enzyme was altered, the phosphorylation site was not modified by the H_2DIDS treatment. The maximum level of phosphorylation by [^{32}P]P_i was the same in normal and H_2DIDS–enzyme, 2.67 ± 0.12 and 2.47 ± 0.14 nmol/mg, respectively.

Location and Quantitation of Modifying Sites. Structural modifications of the enzyme, produced by the H_2DIDS treatment, were examined by using SDS–PAGE. The H_2DIDS–enzyme showed the same protein profile as normal enzyme (Figure 6); no cross-linking of protein fragments was observed. There was no major difference between control and H_2DIDS–enzyme following trypsin treatment in the presence of K^+. However, the peptide profile of H_2DIDS–enzyme treated with trypsin in the presence of Na^+ plus ADP was the same as when the proteolysis was performed in the presence of K^+ (Figure 6). This is in marked contrast to control enzyme, which showed different and characteristic patterns when trypsin treatment is performed with Na plus ADP or with K (Figure 6). Na^+ plus ADP stabilizes E_1 forms while K^+ stabilizes E_2 forms of the enzyme.[3]

The profile of [^{3}H]H_2DIDS labeling shows that only the α-subunit of the enzyme binds H_2DIDS (Figure 6). After trypsin treatment in the presence of either Na^+ plus ADP or K^+ the majority of the radioactivity was found in a fragment that migrated just below the β-subunit.

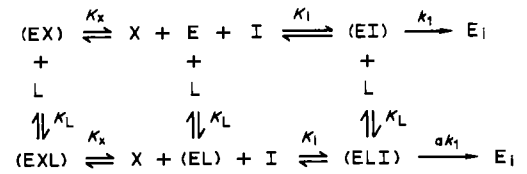
pH Dependence of Inhibitor–Protein Interactions. Isothiocyanate groups react quite specifically with side-chain amino groups of proteins. The reaction can take place, however, only with the deprotonated form of the amine. At pH 9 the inactivation of the Na,K-ATPase was slightly lower than that observed at pH 7.2 (Figure 7). The covalent [^{3}H]H_2DIDS binding was twice as high at pH 7.2 than at pH 9. A plot of residual enzyme activity versus bound H_2DIDS indicates a linear relationship at both pH values. Total inactivation is attained when 3.4 and 6 nmol of H_2DIDS/mg of protein are bound to the enzyme at pH 9 and 7.2, respectively.

Discussion

The present studies show that H_2DIDS and similar compounds can cause both reversible inhibition and inactivation of the sodium pump. It has been shown previously that disulfonic stilbenes inhibit the Na pump (Teisinger et al., 1984;

[3] E_1 is the enzyme form with high affinity for ATP (K_d = 0.1 μM) and Na^+ ($K_{0.5}$ ~ 1 mM) while E_2 has low affinity for ATP ($K_{0.5}$ ~ 0.1 mM) and high affinity for K^+ (K_d ~ 0.3 mM) (Glynn & Karlish, 1975).

Scheme I



Faelli et al., 1984; Ehrenspeck & Brodsky, 1976). These studies suggested that the inhibition took place at the cytoplasmic face of the enzyme. In human erythrocytes, inhibition of anion transport (mediated by band 3) occurs via binding of these compounds to the extracellular surface (Jennings, 1982). Even though the present experiments were made with purified enzyme, DIDS has been observed to have no effect on the red cell sodium pump when added to the external medium (Hoffman et al., 1979). Thus, it is likely that the inhibitory effects observed are occurring at sites on the protein that are normally exposed at the cytoplasmic surface.

Reversible Inhibition. While the reversible inhibition of the Na,K-ATPase by H_2DIDS could be analyzed by assuming a simple noncompetitive mechanism, this was not the case for the pNPPase activity (Figure 1). As far as we know, this is the first time that an inhibitor has been described that affects the pNPPase more than the Na,K-ATPase activity. This indicates that either different sites of interaction between the enzyme and H_2DIDS are affecting both activities or the same binding affects kinetic steps not shared by both activities. It has been proposed previously that these activities proceed via different pathways (Berberian & Beauge, 1985; Shaffer et al., 1978).

The observation that H_2DIDS reversibly inhibited the enzyme in the presence of saturating concentrations of Na^+, K^+, Mg^{2+}, and ATP indicates that the ligand binding sites are not involved in or modified by the *noncovalent H_2DIDS* interaction. After reversible binding of H_2DIDS, the reactivity of the reagent –NCS groups with side-chain residues on the protein will depend on the alignment, distance between the reactive groups, and the local pH. Therefore, the protecting ligands (K^+ and ATP) must produce some change in the accessibility or orientation of the side-chain groups that are involved in covalent bond formation. The higher affinity for K^+ and lower affinity for ATP in protecting against inactivation, compared to the affinity usually observed for those ligands binding the E_1 form of the enzyme, suggest that the reversible H_2DIDS interaction holds the enzyme in an E_2 form. Taking into account this change of conformation and the observation that DNDS prevents the inactivation by H_2DIDS, the simple mechanism of inhibition and protection of the enzyme shown in Scheme I can be considered, where E is the free enzyme; (EX), (EXL), (EI), and (ELI) are the reversibly inhibited enzyme forms, and E_i represents the inactive enzyme. X refers to DNDS; I, to H_2DIDS; and L to K^+, ATP, Mg^{2+}, and Na^+. The value of α is greater than 1 for Na^+ and 0 for K^+ and ATP. Scheme I assumes that DNDS and H_2DIDS are mutually exclusive simple noncompetitive inhibitors with respect to L. The excellent fit of the experimental data with equations derived from this scheme indicates that it is at least compatible with the experimental observations.

Protection by Monovalent Cations. K^+ counteracts the H_2DIDS inactivation of the enzyme with a high affinity (Figure 3) usually associated with binding to the E_2 form of the enzyme (Karlish, 1980; Garrahan & Glynn, 1967; Rephaeli et al., 1986). One interesting feature of the concentration dependence of the first phase of K^+ protection (Figure 3A) was the hyperbolic relation, suggesting that there was only one K^+ ion that initially interacted with the protein and this was sufficient to halt the H_2DIDS inactivation process. Therefore, at maximum K^+ protection there seems to be one K^+ ion binding per enzyme molecule. On the other hand, the biphasic characteristic of the K^+ effect suggests that the binding of a second K^+ ion to the enzyme reduces the protective effect displayed by the binding of the first K^+. Thus, the two K^+ ions bind to each enzyme molecule with very different affinities, producing opposite effects on the H_2DIDS inactivation. This suggests that the binding of K^+ to either one of its two sites produces structural modifications of the enzyme. Recently, evidence for two different K^+ sites has been presented from direct studies of Rb^+ occlusion by the enzyme (Shani et al., 1987). It is noteworthy that the two K_{Rb} measured by Rb^+ binding (Shani et al., 1987) were similar to the half-maximal effects of K^+ on the H_2DIDS inactivation (Figure 3).

The fact that the K^+ protection and Na^+ facilitation of the H_2DIDS inactivation were produced by the binding of only one of either ion to the protein (Figures 3 and 4) indicates that binding of only one K^+ or Na^+ ion per enzyme molecule produces significant modifications in the enzyme structure. In enzyme labeled with FITC Karlish (1980) also observed that the binding of one K^+ ion converted the enzyme from the E_1 to the E_2 conformation and one Na^+ ion returned it to E_1.

The reduced ADP (and, hence, ATP) binding to the high-affinity nucleotide site following inactivation of the enzyme by H_2DIDS is sufficient alone to account for loss of Na,K-ATPase activity. The fact that H_2DIDS–enzyme did not bind ADP might imply that H_2DIDS has reacted with an amino acid residue at the ATP binding site. This would be supported by the observations that (i) ATP counteracted the inactivation (Figure 5), (ii) H_2DIDS–enzyme showed reduced binding of FITC (data not shown), which has been assumed to bind to the ATP site (Karlish, 1980), and (iii) K^+, which has been shown to compete with the binding of ATP (Kaniike et al., 1973), also opposes the inactivation of the enzyme by H_2DIDS. However, similar arguments can be used to support the idea that H_2DIDS is an affinity label for the K^+ binding site. Thus, some caution is necessary in interpreting protection against inactivation or binding with the identification of a site. Protection by a substrate against inactivation of an enzyme does not provide unambiguous evidence that the substrate and inactivating agent bind to the same site.

Comparison with FITC-Modified Protein. Since FITC and H_2DIDS are both amino-reactive reagents, it is possible that the same amino acid is modified. Indeed, prior treatment of the enzyme with H_2DIDS greatly reduces the FITC binding. Furthermore, DNDS, which protects the enzyme against H_2DIDS binding and inactivation, also prevented FITC binding. There are, however, significant differences between the effects of modification by FITC and H_2DIDS (Karlish, 1980; Sen et al., 1981; Pedemonte & Kaplan, 1986a–c; Carilli et al., 1982). It will be of interest to determine if these differences and similarities are the consequence of modification of the same or different amino acid residues on the enzyme α-subunit by the two reagents.

The reaction between H_2DIDS and the Na,K-ATPase appears to involve at least two distinct steps: a rapid reversible interaction and a subsequent slower covalent bond formation between the –NCS groups of H_2DIDS and an amino acid residue on the protein. This mode of action is very similar to that described in the DIDS inhibition of anion transport mediated by band 3 in red blood cells (Lepke et al., 1976; Jennings, 1982; Jennings & Nicknish, 1985). The idea that

the initial reversible interaction determines the subsequent covalent reaction between H_2DIDS and the protein is supported by the effects of pH (see below) and the observation that DNDS, which can only interact reversibly with the protein, protected the enzyme against inactivation by H_2DIDS.

Effects of pH. Raising the pH of the assay medium containing H_2DIDS and the enzyme reduces the reversible inhibition but has no major effect on the inactivation reaction (Figure 7). This suggests that positively charged group(s) of the protein that interact Coulombically with the sulfonate groups of H_2DIDS, resulting in *reversible* inhibition of the enzyme, are deprotonated when the pH is raised from 6.5 to 8.4, resulting in a reduction of inhibition. In contrast, the inactivation of the enzyme is only slightly reduced between pH 7.2 and 9. The side-chain group with which the –NCS of H_2DIDS reacts covalently to produce the inactivation should be at least partially deprotonated at pH 7.2. Therefore, inhibition of the enzyme and covalent binding of H_2DIDS produced by elevation of the pH are due to differences in the initial reversible interaction between H_2DIDS and the protein and not to effects on the subsequent covalent reaction.

Stoichiometry and Localization of Binding. Kinetic studies showed a linear relationship between inactivation of the enzyme and both time of treatment with H_2DIDS and amount of inhibitor binding (Figures 2 and 7). The values of [^{3}H]-H_2DIDS labeling and maximal phosphorylation of the enzyme indicate that the binding of one H_2DIDS molecule per enzyme molecule, at pH 9, causes complete inactivation. A linear relationship is found between H_2DIDS bound and remaining activity at both pH 7.2 (where two H_2DIDS molecules bind at complete inactivation) and pH 9 (where only one H_2DIDS molecule is sufficient for total inactivation). Thus, the second site modified at pH 7.2 may or may not be related to enzyme inactivation.

The profile of radioactivity in SDS–PAGE showed that H_2DIDS was bound to a 52-kDa fragment which has been assumed to include the ATP site (Carilli et al., 1982). In the presence of Na^+ plus ADP the proteolysis pattern of H_2DIDS–enzyme is similar to that usually observed for the K^+ form of the enzyme (E_2 form).

There is no direct evidence to identify which amino acid residues are involved in the H_2DIDS binding domain. The product of the reaction between –NCS and SH groups would not be sufficiently stable (in the labeled enzyme washing conditions) to produce the inactivation observed. Thus, the inactivation is likely due to interaction of H_2DIDS with a side-chain amino residue on the protein. If NH_2-lysine residues are indeed involved in the covalent reaction, they have low p*K*'s, presumably due to either (i) the juxtaposition of other positively charged residues, which favor deprotonation at a lower pH than that expected for an ϵ-NH_2-lysine (p*K* 10.5), or (ii) the fact that the lysine residues are contained in a relatively hydrophobic environment.

The present results show that H_2DIDS is a very useful reagent for identifying functionally important amino acid residues in the Na,K-ATPase. The characteristics of the inactivation and the protection by ATP and K^+ suggest that these two ligand sites may be considered potential binding domains for H_2DIDS. Identification of the specific amino acid residue modified will require the purification and sequencing of the H_2DIDS-labeled peptide fragment.

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Registry No. ATPase, 9000-83-3; H_2DIDS, 61481-03-6; ATP, 56-65-5; K, 7440-09-7; Na, 7440-23-5.

REFERENCES

Askari, A., & Huang, W. (1982) *Biochem. Biophys. Res. Commun. 104*, 1447–1453.

Berberian, G., & Beauge, L. (1985) *Biochim. Biophys. Acta 821*, 17–29.

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

Cantley, L. C., Jr., Gilles, J., & Josephson, L. (1978) *Biochemistry 17*, 418–425.

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

DePont, J. J. H. H. M., Schoot, B. M., Van Prooijen-Van Eden, A., & Bonting, S. L. (1977) *Biochim. Biophys. Acta 482*, 213–227.

Dixon, M., & Webb, E. C. (1979) *Enzymes*, 3rd ed., pp 337–339, Academic, New York.

Ehrenspeck, G., & Brodsky, W. (1976) *Biochim. Biophys. Acta 419*, 555–558.

Faelli, A., Tosco, M., Orsenigo, M. N., & Esposito, G. (1984) *Pharmacol. Res. Commun. 16*, 339–350.

Farley, R. A., Tran, C. M., Carilli, C. T., Hawke, D., & Shively, J. E. (1984) *J. Biol. Chem. 259*, 9532–9535.

Garrahan, P. J., & Glynn, I. M. (1967) *J. Physiol. (London) 192*, 175–188.

Glynn, I. M., & Karlish, S. J. D. (1975) *Annu. Rev. Physiol. 37*, 13–54.

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

Hoffman, J. F., Kaplan, J. H., & Callahan, T. J. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 2440–2441.

Jennings, M. L. (1982) *J. Biol. Chem. 257*, 7554–7559.

Jennings, M. L., & Nicknish, J. S. (1985) *J. Biol. Chem. 260*, 5472–5479.

Jorgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 36–52.

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

Kaniike, K., Erdmann, E., & Schoner, W. (1973) *Biochim. Biophys. Acta 298*, 901–905.

Kaplan, J. H. (1983) *Am. J. Physiol. 245*, G327–G333.

Kaplan, J. H. (1985) *Annu. Rev. Physiol. 47*, 535–544.

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

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

Kenney, L. J., & Kaplan, J. H. (1985) in *The Sodium Pump* (Glynn, I. M., & Ellory, J. C., Eds.) pp 535–539, The Company of Biologists, Cambridge, England.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lepke, S., Fasold, H., Pring, M., & Passow, H. (1976) *J. Membr. Biol. 29*, 147–177.

Liang, S. M. L., & Winter, C. G. (1976) *Biochim. Biophys. Acta 452*, 552–565.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Ohta, T., Nagano, K., & Yoshida, M. (1986) *Proc.* Natl. *Acad. Sci. U.S.A. 83*, 2071–2075.

Patzelt-Wenczler, R., & Schoner, W. (1981) *Eur. J. Biochem. 114*, 79–87.

Pedemonte, C. H., & Beauge, L. A. (1986) *Arch. Biochem. Biophys. 244*, 596–606.

Pedemonte, C. H., & Kaplan, J. H. (1986a) *Biophys. J. 49*, 35.

Pedemonte, C. H., & Kaplan, J. H. (1986b) *J. Biol. Chem. 261*, 3632–3639.

Pedemonte, C. H., & Kaplan, J. H. (1986c) *J. Biol. Chem. 261*, 16660–16665.
Pedemonte, C. H., & Kaplan, J. H. (1988) *Prog. Clin. Biol. Res. 268A*, 327–334.
Rao, A., Martin, P., Reithmeier, A. F., & Cantley, L. C. (1979) *Biochemistry 18*, 4505–4516.
Rephaeli, A., Richard, D., & Karlish, S. J. D. (1986) *J. Biol. Chem. 261*, 6248–6254.
Robinson, J. D. (1980) *J. Bioenerg. Biomembr. 12*, 165–174.
Schneider, J. W., Mercer, R. W., Caplan, M., Emanuel, J. R., Sweadner, K., Benz, E. J., Jr., & Levenson, R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6357–6361.
Segel, I. H. (1975) *Enzyme* Kinetics, pp 125–135, Wiley, New York.
Sen, P. C., Kaparos, J. G., & Steinberg, M. (1981) *Arch. Biochem. Biophys. 211*, 652–661.
Shaffer, E., Azari, J., & Dahms, A. S. (1978) *J. Biol. Chem. 253*, 5696–5706.
Shani, M., Goldschleger, R., & Karlish, S. J. D. (1987) *Biochim. Biophys. Acta 904*, 13–21.
Shull, G. E., Schwartz, A., & Lingrel, J. B. (1985) *Nature (London) 316*, 691–695.
Skou, J. C. (1979) *Biochim. Biophys. Acta 567*, 421–435.
Teisinger, J., Zemkova, H., & Vyskocil, F. (1984) *FEBS Lett. 175*, 275–278.
Walderhaug, M. O., Post, R. L., Saccomani, G., Leonard, R. T., & Briskin, D. P. (1985) *J. Biol. Chem. 260*, 3852–3859.

Interactions of Proteins with Ganglioside-Enriched Microdomains on the Membrane: The Lateral Phase Separation of Molecular Species of GD1a Ganglioside, Having Homogeneous Long-Chain Base Composition, Is Recognized by *Vibrio cholerae* Sialidase†

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ABSTRACT: The thermotropic behavior (studied by high-sensitivity differential scanning calorimetry) and susceptibility to *Vibrio cholerae* sialidase hydrolysis of large unilamellar vesicles of dipalmitoylphosphatidylcholine, containing native GD1a ganglioside or the molecular species of GD1a containing C18:1 or C20:1 long-chain base (C18:1 GD1a; C20:1 GD1a), were studied. Vesicles containing ganglioside (10% in molar terms) showed the presence in the heat capacity function of a second minor peak besides the phospholipid main transition peak. The presence of a second peak is much more evident with C20:1 GD1a than with C18:1 GD1a, the difference being potentiated by Ca^{2+} and indicating a different tendency of the GD1a molecular species to undergo lateral phase separation. The scans of vesicles containing native GD1a showed the features of those obtained with C18:1 GD1a and C20:1 GD1a, indicating that the main components of native GD1a, C18:1 GD1a and C20:1 GD1a, maintain their individual aggregative properties. *V. cholerae* sialidase affects vesicle-bound GD1a at a much higher rate (17–25-fold) than it does micellar GD1a, the activation by Ca^{2+} being 3- and 2-fold, respectively. The V_{max} values were identical on C18:1 GD1a and C20:1 GD1a in micellar dispersions, whereas they were markedly higher (from 20 to 50%) on C18:1 GD1a than on C20:1 GD1a in vesicular dispersions. Exhaustive sialidase hydrolysis of vesicles carrying native GD1a produced C18:1 GM1 and C20:1 GM1 in the same proportion as the C18:1 and C20:1 species present in native GD1a (53.9% and 46.1%). Conversely, sialidase treatment producing about 10% of GD1a hydrolysis gave origin to C18:1 GM1 and C20:1 GM1 in the proportions of 65–69% and 31–35%, indicating the preference by the enzyme to affect C18:1 GD1a. These data show that *V. cholerae* sialidase is able to recognize GD1a molecules with different long-chain base moieties on the basis of their tendency to undergo lateral phase separation on the membrane, realizing higher V_{max} values of the substrate molecules more dispersed on the surface.

Gangliosides are amphiphilic components of the plasma membrane of vertebrate cells and are asymmetrically located in the outer face of the membrane with the oligosaccharide portions that protrude from the cell surface and interact with a variety of external ligands. Gangliosides are known to participate in a number of cell surface events where the oligosaccharide chains seem to be instrumental to functional performance (Sharom & Grant, 1978; Brady & Fishman, 1979; Ando, 1983). The actual availability of the ganglioside oligosaccharide chains to interactions has been shown (Masserini et al., 1982; Myers et al., 1984) to depend on the physicochemical properties of the embedding membrane, as well as on the aggregative properties of the ganglioside molecules. In turn, these latter properties are largely determined by the chemical characteristics of the ganglioside lipidic portion (ceramide) which is inserted into the lipid bilayer, intercalating with the other hydrophobic components of the membrane

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