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ENTITLED THE EFFECTS OF MEMBRANE DOMAIN FORMATION AND ELECTROSTATIC

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THE EFFECTS OF MEMBRANE DOMAIN FORMATION AND ELECTROSTATIC FORCES ON THE ELECTRON TRANSPORT CHAIN OF E. COLI

BY

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Abbreviations

PA - phosphatidic acid
PS - phosphatidyl-serine
NADH - nicotinamide adenine dinucleotide; reduced form
d-NADH - deamino nicotinamide adenine dinucleotide; reduced form
LB - Luria-Bertani medium
EDTA - ethylene diaminotetraacetic acid
Tris - tris (hydroxymethyl) aminomethane
BSA - bovine serum albumin
DCIP - dichlorophenol indophenol
TLC - thin-layer chromatography
AMP - adenosine monophosphate
CTP - cytosine triphosphate

Introduction

One basic tenet of the Fluid Mosaic Model for membrane structure is that the lipid and protein components of the membrane are free to diffuse laterally in the bilayer in a random fashion (1). However, there has been an accumulation of evidence, some of which has been provided by this laboratory, that suggests the existence of regions of long-range order in some membranes. These regions are often referred to as "patches" or "domains". The evidence for membrane domain formation has come from studies on many different systems, including both biological and artificial ones. An overview of this research is provided by Rodgers (2).

Most importantly for my project, it has been found that divalent cations, specifically calcium, strongly stimulate the formation of domains in membranes that contain significant levels of acidic phospholipids, such as PA or PS (3,4,5). Most of these studies were done using methods such as differential scanning calorimetry or electron spin resonance to detect the formation of domains. Techniques have also been developed in this laboratory to observe these patches directly with flourescence imaging microscopy. The formation of PA-enriched domains in artificial vesicles exposed to 2 mM calcium chloride has been visualized using this method (6).

The main goal of this research project, then, is to investigate the effects of this calcium-induced "patching" of membranes on a membrane-bound enzyme, as an example of the functional significance of domains. E. coli NADH dehydrogenase was chosen as the protein because it is easily assayed and present in relative abundance on the inner membrane.

NADH dehydrogenase is actually a generic term for an enzyme that utilizes the reducing power of NADH to reduce some other substrate. However, the NADH dehydrogenase that is especially interesting to study is the first enzyme in the electron transport chain of E. coli (7). It transfers electrons from NADH to the next carrier in the electron transport chain. The chain as a whole eventually reduces molecular oxygen to water. The activity of the

electron transport chain can be inhibited by cyanide.

When this occurs, an alternate pathway for the electrons of NADH can be utilized by providing an artificial electron acceptor such as DCIP to the system.

A mutant strain of E. coli called GN80 was available that had a pH-sensitive, conditionally lethal mutation in its cds locus. When this protein is "knocked out" by elevating the pH of the cellular medium to 8.5, CTP-phosphatidic acid cytidyltransferase, the enzyme that converts PA to CDP-diglyceride in the lipid biogenesis pathway, is no longer active (8). PA therefore accumulates in the membranes of these cells. Thus, since vesicles can be prepared from these membranes, a system is created in which the electron transport chain is in a membrane bilayer that contains unusually high levels of an acidic phospholipid. (PA is about 30 mole% rather than the 0.5 mole% found in wild type.) Calcium was added to stimulate domain formation, and the effects of this addition on the kinetics of NADH dehydrogenase were observed.

A complication inherent in this system arises from the fact that NADH dehydrogenase is surrounded by a membrane environment that is highly negatively charged. Its substrate, NADH, the disappearance of which is the basis for the enzyme's assay, is also negatively charged. Therefore one would expect a change in the behavior of the enzyme from "wild-type vesicle" to "mutant vesicle" based simply on the presence of additional electrostatic forces in the mutant. Furthermore, the addition of divalent calcium would probably electrostatically "screen" these forces and again affect the kinetics of the enzyme, in addition to whatever effect the patching would have. To correct for the electrostatic effects, sodium was added in separate experiments, and its effects on the enzyme's kinetics were also observed.

It is important to note, however, that the effect of electrostatic forces on a membrane enzyme's behavior is not completely understood either, and is of interest in itself (9).

The work that has been done on this project is readily divided into two areas. The first area of research involves the preliminary kinetics studies of calcium and sodium addition to the high-PA GN80 vesicles. The results of this research will be presented here. It will be seen, however, that the system used had to be further characterized and simplified in order to truly identify the effects of domain formation and electrostatics on the membrane enzyme's behavior. This was the major goal of the second part of the research.

Materials and Methods - I

Bacterial Strains GN80 and the isogenic wild type GN85 E. coli strains were generously donated by Dr. Raetz of the University of Wisconsin-Madison (8).

Materials All chemicals used were reagent grade.

Growth of Bacteria Permanent stocks of GN80 and GN85 were stored at -70 °C. Temporary streak-cultures were grown at 37 °C and stored at 4 °C on LB-agar plates. For vesicle preparations, cells were grown in H56 media, the composition of which is described by Raetz (8). Cell culture turbidity was monitored by O.D. 600 nm with a Beckman spectrophotometer. If the pH of the growth media was changed during cell growth, it was monitored with an Orion Research pH-meter and altered with sterile 2N KOH.

Preparation of Inner Membrane Vesicles Vesicles of E. coli inner membrane were prepared by the method of Osborn (10). This method basically consists of treating resuspended cells with lysozyme and EDTA to create spheroplasts, and then sonicating to create vesicles. Inner membrane vesicles are then isolated using sucrose-density centrifugation. Sonication was done with 30 second pulses on an Ultrasonics, Inc. W-375 sonicator. Centrifugations were performed with an L5-50 Beckman ultracentrifuge. Inner membrane vesicle pellets were resuspended in 10mM Tris, pH 7.5 and stored in freezer vials in liquid nitrogen.

Protein Determination Protein concentrations were determined by the method of Lowry with BSA as a standard (11).

Enzyme Assays NADH dehydrogenase was assayed as a DCIP-reductase by monitoring the reduction of DCIP at 600 nm (12). The assay mixture contained 10 mM Tris-Cl, 10 mM KCN, 32 uM DCIP,

vesicles (with enzyme), and NADH in a final volume of 500 uL. The decrease in optical density was monitored by a Beckman spectrophotometer equipped with a strip-chart recorder. Activity values were corrected for reduction of DCIP in the absence of added vesicles. NADH oxidase activity was similarly measured by monitoring the decrease in optical density at 340 nm due to the oxidation of NADH. The assay mixture was the same as above with the omission of KCN.

For specific activity measurements, NADH was present at 250 uM concentrations in these assays. For Km determinations, the linear range was determined first by varying the amount of protein (i.e. volume of vesicles added) at this NADH concentration. Then the NADH concentration was varied at a fixed protein amount. Assays were initiated by adding the vesicle solution to the assay mixture.

Most assays were conducted at 15 °C because the reactions were found to proceed too quickly for accurate monitoring at room temperature. This was done by incubating cuvettes with assay mixture in a 15 °C constant temperature bath before vesicle addition. The spectrophotometer itself, however, was at ambient temperature.

The terms NADH DCIP-reductase and NADH dehydrogenase are used equivalently in this thesis.

Enzyme Assay Data Analysis Kinetic data was analyzed in several different ways, but the main method of analysis was curve-fitting to a simple Michaelis-Menten model. This was done using a computer program called HYPER designed by Dr. W. Wallace Cleland (13). This program generates values for the apparent Km and Vmax of the enzyme, as well as estimates of the error inherent in these values. The extinction coefficients used were 16.5 mM -1 cm -1 for DCIP and 6.22 mM -1 cm -1 for NADH.

Results - I

The preliminary studies of the effects of calcium and sodium addition on NADH dehydrogenase and oxidase kinetics in high-PA vesicles are summarized in Table I. In general, the addition of 50 or 100 mM NaCl lowered the Km (NADH) of the enzyme about two-fold over no-addition controls, with the maximal velocity remaining fairly constant. The addition of 0.5 or 10 millimolar calcium chloride lowered the Km to a lesser degree. A control experiment was performed by William Rodgers in which similar experiments were done on GN80 vesicles that had been pH-shifted during growth for much less time before harvesting, i.e., were presumably much closer to wild-type PA levels (8). In these experiments the 50 mM sodium ions, as well as the 0.5 mM calcium ions, had little or no effect on the Km (NADH) over no-addition controls. (see Table 2) Results of a typical assay of high-PA vesicles are shown on a Lineweaver-Burke plot in Figure 1. It is important to note that these assays have a relatively high level of error or deviation associated with them. Despite efforts to control this error, most assays routinely yielded Km values with 30-50% relative standard deviation.

Although the NADH oxidase activity trends shown in Table I are somewhat similar to those of the DCIP-reductase activity, they involve even more fluctuation and error than the dehydrogenase assays, especially with regards to maximal velocity.

It appeared that the sodium and calcium additions were indeed having some effect on the dehydrogenase kinetics, but these effects were difficult to discern because of the high degree of error involved. The research done towards identifying the sources of error and eliminating them in the system to clarify our results is described in the second section of this manuscript.

Materials and Methods - II

Bacterial Strains GN80 and GN85 as described previously. MWC003, a exo⁻ E. coli strain was generously donated by the Gennis laboratory. A tetracycline resistance marker is associated with the exo gene in this strain.

Growth of Bacteria For cultures of GN80 that were not to be pH-shifted, LB-broth was used as the medium for growth.

Membrane Vesicles For some experiments, it was decided that inner membrane vesicles were not required. In these cases, the purification procedure of Osborn described previously was stopped before the sucrose-density gradient centrifugation step. This mixture of vesicles was pelleted and resuspended in 10 mM Tris, pH 7.5 and stored in freezer vials in liquid nitrogen.

French Press The French-press method of breaking cells was tried and abandoned as an alternative to sonication. Using an Amicon apparatus, the cells were broken by pressure at about 1100 psi, and the effluent collected.

Thin Layer Chromatography of Phospholipids The Raetz one-dimensional TLC system was used to separate phospholipids (8). Lipids were extracted from whole cells by the method of Bligh and Dyer (14). Analtech silica gel G, 5 x 20 centimeter, TLC plates were used. After separation and overnight air-drying, the separated lipids were visualized by iodine staining.

Strain Construction Plcml,clr100 phage was generously donated by Lee Bussey. This phage has an associated chloramphenicol resistance marker which it confers upon the bacterium it lysogenizes (15). Lysate production and transduction with this phage was carried out as described by Miller (15). Successful transductants were detected by growth on LB-broth agar plates containing 12.5 ug/mL of tetracycline.

Enzyme Assays Assays of NADH DCIP-reductase and NADH oxidase activity were performed as described previously. In addition, deamino-NADH oxidase and NADH DCIP-reductase activities were also measured in the same way, with deamino-NADH obtained from Sigma substituted for the standard NADH.

Results - II

From the results of the preliminary kinetics studies, it appeared that it was neccesary to reduce or eliminate the sources of error in the assays in order to better interpret the data that was obtained. There were several possible targets for such an approach. One possibility was that assay conditions such as temperature, substrate concentrations, etc. were not being stringently enough controlled. Another possibility was that the enzyme activity was being lost during vesicle preparations to varying degrees, either through denaturation or physical separation. A very low amount of enzyme activity that fluctuated relatively significantly between preparations could be a source of assay error. This was suspected because the NADH dehydrogenase specific activities reported in the literature using similar vesicle preparation methods were consistently 5-10 times greater than the specific activities that were measured (12,16).

Finally, a third possibility was that the enzyme system that was being assayed was more complicated than initially hoped, and that one or more unidentified enzymes with DCIP-reductase activity were also being assayed at the same time.

Since attempts at more rigidly controlling the assay conditions did not seem to affect the results, a thorough study of the enzyme's activity throughout the vesicle preparation procedure was performed. A culture of GN80 cells was taken through the steps in the inner membrane vesicle preparation, with aliquots of material removed from the bulk solution at various points in the procedure. These aliqouts were either assayed immediately for NADH dehydrogenase activity or assayed after three days at 4 degrees C or in liquid nitrogen. This was done to gauge the temporal stability of the activity for different storage conditions. The results of this study are shown in Table 3.

From these studies, it appeared as though the time-consuming sucrose-density gradient centrifugation step was unnecessary to increase the specific activity of the enzyme, and that the "after

sonication" vesicles could be used as a source of maximal specific activity. However, from other studies, it was suspected that the activity in this fraction may be due to some soluble diaphorase rather than the membrane-bound, electron transport chain-linked NADH dehydrogenase (12). To test this suspicion, AMP inhibition experiments were perfomed on this fraction. Both the oxidase and the dehydrogenase activities of this sample were inhibited about 50% by 10 mM AMP, indicating that the activities were due mainly to the membrane-bound dehydrogenase. (see table 4) In addition, the measured Km (NADH)'s for the oxidase and dehydrogenase activities were within experimental error of each other (approximately fifteen micromolar) , again suggesting the lack of a significant population of soluble DCIP-reductases with different Km (NADH)'s (12).

However, after additional kinetics studies with this new, shortened vesicle preparation exhibited problems similar to the older studies, it was decided that maximizing the specific activity was not going to solve the problem of assay precision.

Further analysis of this and previous data with Eadie-Hofstee plot constructions, which are especially suited to studying linearity in enzyme assays, revealed a biphasicity in much of the data that pointed to a multi-enzyme problem. (see Figure 2 for details) Upon further reference to the literature, it seemed probable that a second, membrane-bound, respiratory-chain NADH dehydrogenase called NADHdh1 (cxo gene locus) was responsible for this biphasic behavior (17). Eliminating this enzyme could, therefore, increase the precision and accuracy of the enzyme assays.

Although this enzyme has not been completely characterized at this point, it has the properties of being able to use both NADH and deamino-NADH as substrates and is a proton pump. The NADH dehydrogenase that we wish to study, mainly because it is better characterized and purified, (gene locus ndh) cannot utilize deamino-NADH as a substrate and is not a proton pump (17).

Since researchers in the Gennis laboratory had obtained an E. coli strain MWC003 that had the cxo gene "knocked out" and assoc-

iated with a tetracycline resistance marker, Dr. Glaser suggested that I could introduce this mutation by homologous recombination into the GN80 strain via P1 viral transduction. Therefore, the MWC003 strain was infected with the P1 phage and the successfully infected cells were lysogenized on LB-chloramphenicol plates at 32 °C. Chloramphenicol was present at 15 ug/ml in these plates. A lysate was subsequently prepared by culturing a colony off of this lysogen plate and raising the liquid culture temperature to 40 °C for a period of time. The lysate was separated from the dead cells by a brief centrifugation. This lysate was then used to infect GN80 cells in culture. The GN80 cells were later plated out onto LB-tetracycline agar plates in order to detect successful transductants. The details of this procedure are given in Miller, as stated previously.

Characterization of the lone successful transduction as detected by tetracycline resistance (i.e. testing for the cxo mutation) was performed in two ways. The colony, named GN81, was assayed for loss of deamino-NADH DCIP-reductase activity. The results of this assay are shown in table 5. Ability to use this substrate was completely lost. In addition, the propagated colony was tested to make sure that it retained the pH-sensitive cds mutation by TLC of whole cell lipids after pH-shifting of growth media. Both the GN80 and GN81 control lanes exhibited nearly identical patterns of phospholipid staining, with a prominent high-mobility spot attributable to PA.

The GN81 was cultured and stored at -70 °C in a 15% glycerol solution for long-term storage.

Table 1 - Kinetic Assays of High-PA (100 minute pH-shift) GN80 vesicles @ 15 °C

Assay	Additions	Km (NADH)	Vmax
dehyd.	none	13.1 +/- 3.4 uM	0.052 nmoles/min.ug
dehyd.	50 mM NaCl	6.5 +/- 3.2 uM	0.053 " " "
dehyd.	100 mM NaCl	5.5 +/- 1.9 uM	0.033 " " "
dehyd.	0.5 mM CaCl	9.0 +/- 1.5 uM	0.048 " " "
dehyd.	10 mM CaCl	12.1 +/- 2.2 uM	0.053 " " "
oxidase	none	27.3 +/- 18.1 uM	0.301 " " "
oxidase	50 mM NaCl	23.3 +/- 12.6 uM	0.081 " " "
oxidase	0.5 mM CaCl	62.6 +/- 26.1 uM	0.412 " " "
oxidase	10 mM CaCl	25.9 +/- 6.3 uM	0.101 " " "

Velocities, in general, had an error estimate of about 0.005 nanomoles per minute per microgram.

Table 2 - Kinetic Assays of Low-PA (30 minute pH-shift) GN80 vesicles @ 15 °C

Assay	Additions	Km (NADH)	Vmax
dehyd.	none	3.8 +/- 0.6 uM	0.018 nmoles/min.ug
dehyd.	50 mM NaCl	4.5 +/- 0.9 uM	0.019 " " "
dehyd.	0.5 mM CaCl	2.7 +/- 0.6 uM	0.019 " " "

Table 3 - Variation in DCIP-Reductase Specific Activity

Sample	Assayed Immediately	3-Day at 4 °C	3-Day in Liq. N
Whole Cells	0.018	0.018	0.017
Before Sonication	0.222	0.102	0.277
After Sonication	0.320	0.146	0.254
Inner Membrane Vesicles	0.316	0.189	0.153

Activity values given in units of micromoles per minute per milligram of protein.

Table 4 - AMP Inhibition Studies with NADH Dehydrogenase and Oxidase

NADH Dehydrogenase Sp. Activity: no AMP -- 0.084
10 mM AMP -- 0.047
(44% inhibition)

NADH Oxidase Sp. Activity: no AMP -- 0.332
10 mM AMP -- 0.166
(50% inhibition)

Activities given in micromoles per minute per milligram of protein.

Table 5 - NADH and d-NADH Oxidase Sp. Activities of GN80 and GN81

NADH Oxidase:	GN80 -- 0.72
	GN81 -- 0.98
d-NADH Oxidase:	GN80 -- 0.30
	GN81 -- 0.00

Activities given in micromoles per minute per milligram of protein.

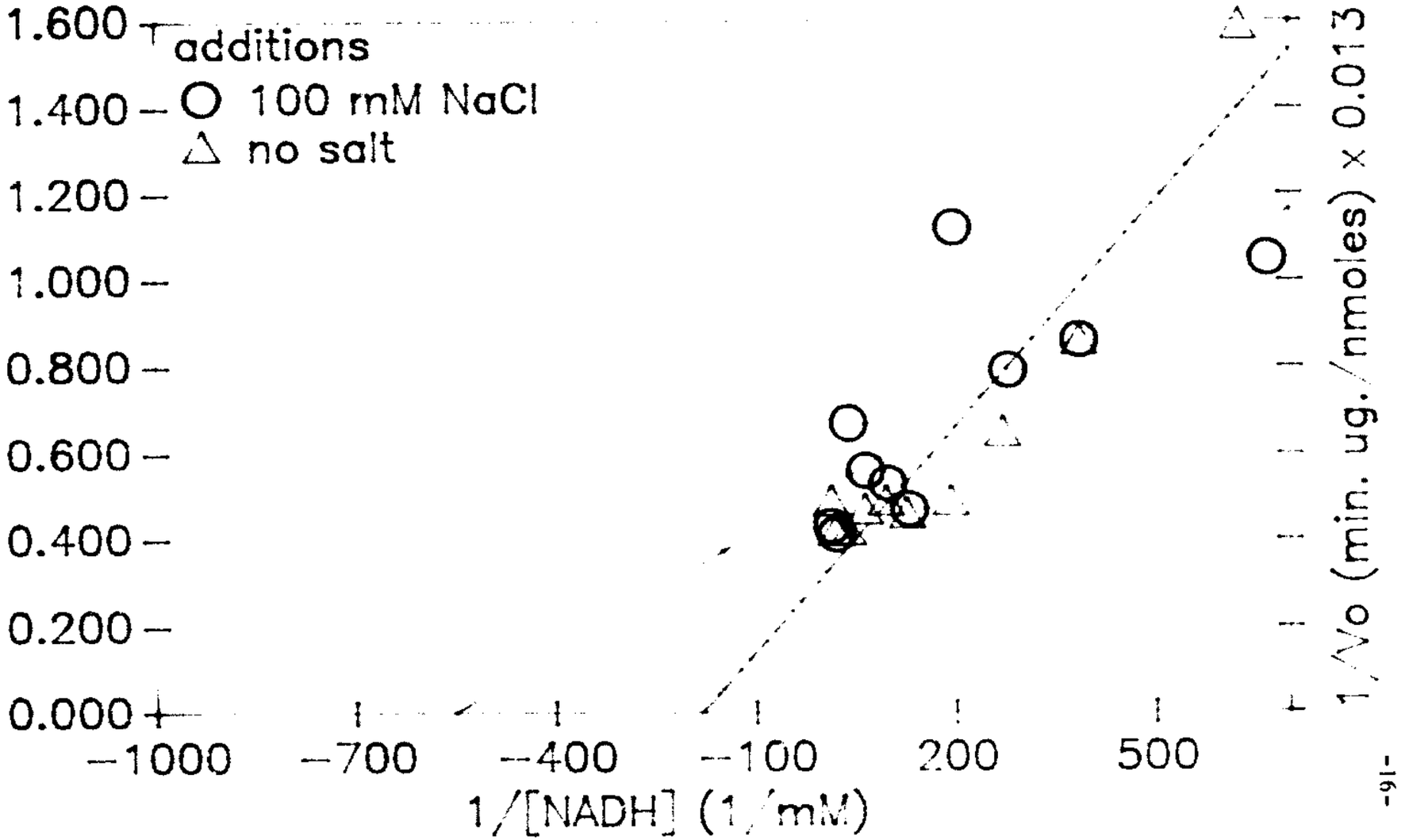

Figure 1. Typical DCIP—reductase Assays

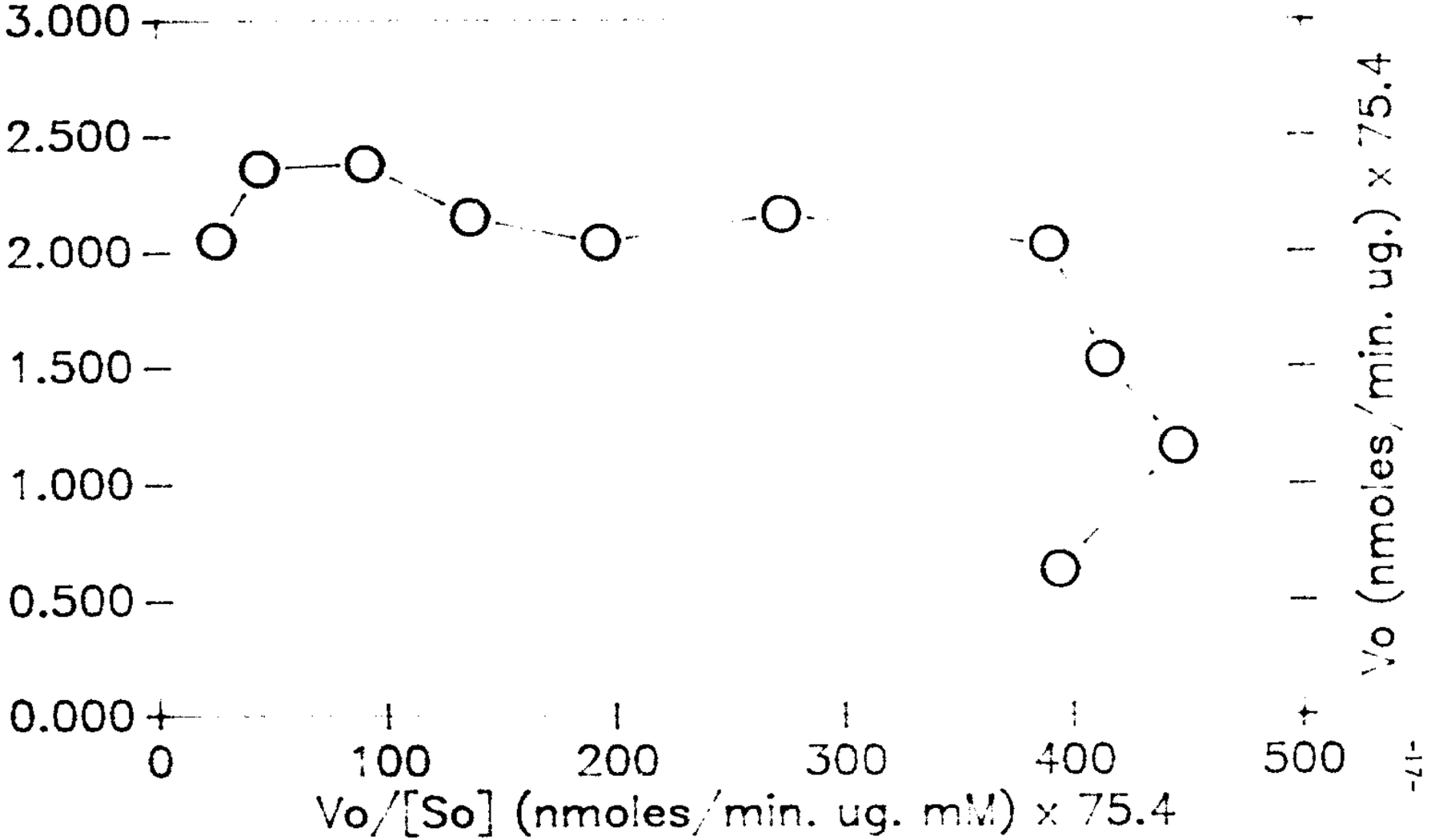

Figure 2. A typical Eadie–Hofstee plot for DCIP–reductase data

Discussion/Conclusions

The addition of cations to high-PA membrane vesicles of E. coli does affect the NADH dehydrogenase activity of this system. The apparent Km (NADH) of the enzyme(s) is significantly reduced in the presence of sodium ions in the 50-100 mM range, while the maximal velocity remains fairly constant. The presence of calcium chloride in the 1-10 mM range also appears to affect the enzyme's kinetic parameters, but in a less clearly-defined fashion.

Since the monovalent sodium ions presumably can not cause domain formation with the acidic phospholipids, their effects are probably mediated solely through electrostatic forces. The reduction of the apparent Km(NADH) of the dehydrogenase, therefore, is evidence for electrostatic screening of the charge-charge repulsions between the acidic phospholipids and the negatively-charged NADH. The theoretical background for this effect is governed by the Gouy-Chapman theory, as described by McLaughlin (18).

The attempts to determine if the effects of calcium on the enzyme are identical to the to the effects of sodium, or are somehow different due to PA-domain formation in the lipid environment of the enzyme, have largely failed. However, steps have been taken towards accomplishing this goal. In particular, the purported construction of GN81, a strain in which an "interfering" enzyme has been eliminated, should help in further studies of this question.

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References

1. Stryer, L. (1988) Biochemistry p.284
2. Rodgers, W., and Glaser, M. (in preparation)
3. Ohnishi, S., and Ito, T. (1974) *Biochemistry* 13, p.881-887
4. Jacobson, K., and Papahadjopoulos, D. (1975) *Biochemistry* 14, p.152-161
5. Ohki, K., Takahashi, K., Kato, S., and Maesano, A. (1989) *Chem. Phys. Lipids* 50, p.109-117
6. Haverstick, D. and Glaser, M. (1988) *J. Cell Biol.* 106, p.1885-1892
7. Jones, C. (1982) Bacterial Respiration and Photosynthesis, Ch.2
8. Ganong, B., and Raetz, C. (1982) *J. Biol. Chem.* 257, p.389-394
9. Clancy, R., Wissenberg, A., and Glaser, M. (1981) *Biochemistry* 21, p.6060-6065
10. Osborn, M., Gander, J., Parisi, E., and Carson, J. (1972) *J. Biol. Chem* 247, p.3962-3972
11. Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951) *J. Biol. Chem.* 193, p.265-275
12. Dancey, G., Levine, A., and Shapiro, B. (1976) *J. Biol. Chem.* 251, p.5911-5920
13. Cleland, W. (1979) *Methods in Enzymology* 63, p.103-138
14. Bligh and Dyer (1959) *Can. J. Biochem. Physiol.* 37, p.911-918
15. Miller, J. H. (1972) Experiments in Molecular Genetics
16. Gutman, M., Schejter, A., and Avi-Dor Y. (1968) *Biochem. Biophys. Acta.* 162, p.506-517
17. Matsushita, K., Ohnishi, T., and Kaback, H. (1987) *Biochemistry* 26, p.7732-7737
18. McLaughlin, S. Electrostatic Potentials at Membrane-Solution Interfaces , p.71-135