Biochemical Pharmacology, Vol 46, No. 8, pp. 1481–1486, 1993
Printed in Great Britain

0006-2952/93 $6.00 + 0 00

BIOCHEMICAL CHARACTERIZATION OF A MEMBRANE-BOUND ENZYME RESPONSIBLE FOR GENERATING NITRIC OXIDE FROM NITROGLYCERIN IN VASCULAR SMOOTH MUSCLE CELLS

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(*Received* 9 *March* 1993; *accepted* 7 *June* 1993)

Abstract—A membrane-bound enzyme responsible for generating nitric oxide (NO) from nitroglycerin (NTG) in vascular smooth muscle cells has been characterized. The enzyme could be solubilized from vascular microsomes by several detergents, the most effective of which was 3-[(3-cholamidopropyl)-dimethylamino]-1-propanesulfonate (CHAPS). A partially purified enzyme preparation was obtained with CHAPS-solubilized vascular microsomes that were processed sequentially through an ion exchange column and a gel filtration column. The activity of this partially purified enzyme showed a dependence on substrate concentration, protein concentration and the duration of incubation. Enzyme activity was enhanced 2.7- to 4.2-fold by several thiols such as cysteine, *N*-acetylcysteine, reduced glutathione, and dithiothretol. On the other hand, *N*-ethylmaleimide, iodoacetic acid, *p*-chloromercuric benzoic acid and 1-chloro-2,4-dinitrobenzene, reagents known to bind with the free sulfhydryl groups, inactivated the NO-generating activity from NTG. The enzyme activity could be reversibly bound to an organomercurial column. These results suggested the presence of a free thiol group in the enzyme and that this thiol group was required for enzyme activity. The partially purified enzyme was active in the presence of 0.1% sodium dodecyl sulfate (SDS). The enzyme was purified to near homogeneity using several sequential chromatographic steps including DEAE-Sephacel, Biogel A 1.5 m, hydroxylapatite and organomercurial columns, resulting in an increase in enzyme activity of about 94-fold. The subunit of this enzyme, as identified on an SDS-treated electrophoresis gel, had an apparent molecular size of 58 kDa.

It is now generally accepted that nitric oxide (NO‡) is the active end-product that is responsible for the pharmacologic action of nitrovasodilators [1]. Recently, we have shown the presence of a membrane-bound enzyme in the bovine smooth muscle cell that appears to be primarily responsible for NO generation from organic nitrate esters [2, 3]. This vascular enzyme is not glutathione-*S*-transferase [4], and is distinctly different from enzymes that metabolically activate organic nitrites [5], *S*-nitrosothiols [6] and sodium nitroprusside [7]. The identity and characteristics of this membrane-bound nitrate metabolizing enzyme, however, have not been examined in detail. Recently, several reports have suggested that a cytochrome P450 related enzyme may be responsible for the process of NO production from organic nitrates, but the cells used in these experiments did not derive from the vascular smooth muscle [8–11]. In this study, we have examined some of the biochemical characteristics of this membrane-bound enzyme using a partially purified preparation obtained from vascular smooth muscle cells. We further purified this enzyme to near homogeneity.

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‡ Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; CHAPS, 3-[(3-cholamidopropyl)-dimethylamino]-1-propanesulfonate; DTT, dithiothreitol; EGTA, ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; IAA, iodoacetic acid; NAC, *N*-acetylcysteine; NADH, β-nicotinamide adenine dinucleotide (reduced form); NADPH, β-nicotinamide adenine dinucleotide phosphate (reduced form); NEM, *N*-ethylmaleimide; NO, nitric oxide; NTG, nitroglycerin; pCMB, *p*-chloromercuric benzoic acid; PMSF, phenylmethylsulfonyl fluoride; SBP, sulfobromophthalein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and SOD, superoxide dismutase.

MATERIALS AND METHODS

Materials

Nitroglycerin (NTG) was obtained from Schwarz Pharma (Monheim, Germany). Superoxide dismutase (SOD) from bovine erythrocytes, phenylmethylsulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), iodoacetic acid (IAA), 1-chloro-2,4-dinitrobenzene (CDNB), sulfobromophthalein (SBP), cysteine (Cys), reduced glutathione (GSH), oxidized glutathione (GSSG), dithiothreitol (DTT), β-nicotinamide adenine dinucleotide (reduced form) (NADH), β-nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH), DEAE-Sephacel and detergents were obtained from the Sigma Chemical Co. (St. Louis, MO). Authentic

NO gas was obtained as 5.7 ppm NO in nitrogen and as pure NO from Linde Specialty Gases (Danbury, CT). Biogel A 1.5m, Affigel 501, hydroxyapatite, silver staining kit and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad (Richmond, CA). All other chemicals were at least of reagent grade.

Preparations of coronary microsomes

Bovine hearts were obtained from a local slaughterhouse. Coronary arteries were removed and smooth muscle cells were obtained as described previously [1]. The cells were homogenized in a buffer containing 250 mM sucrose and 1 mM PMSF. The homogenates were centrifuged at 15,000 *g* for 15 min and the supernatants obtained were recentrifuged at 100,000 *g* for 1 hr. The pellets thus isolated were stored at −70° and used for subsequent purification.

Solubilization of proteins by detergents

Aliquots of coronary microsomes (2 mg protein) were incubated with a 2-mL solution of various detergents at 4° for 1 hr. The concentrations of detergents used were: deoxycholate, 2%; sodium cholate, 2%; Triton X-100, 1%; digitonin, 1%; 3-[(3 - cholamidopropyl) - dimethylamino] - 1 - propanesulfonate (CHAPS), 3%; octyl glucoside, 1%. In addition, microsomes were also incubated with 1 mM KCl, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) or 250 mM sucrose. At the end of the incubation, the samples were centrifuged at 100,000 *g* for 1 hr. The supernatants obtained were directly used for the determination of NO-generating activity.

Preparation of partially purified enzyme

DEAE-Sephacel. Coronary microsomes were first extracted with CHAPS (3%) and the 100,000 *g* supernatant fraction was diluted six times with a buffer containing 20 mM phosphate and 1 mM PMSF to reduce the concentration of CHAPS to 0.5%. The diluted extracts were loaded on a DEAE-Sephacel column (2 × 25 cm) pre-equilibrated with 20 mM phosphate, 1 mM PMSF and 0.5% CHAPS (buffer A). After washing the column with 200 mL of this buffer, the proteins were eluted using a salt gradient of buffer A containing 0–400 mM NaCl. Fractions of 5 mL each were collected, and their optical density at 280 nm (O.D. 280) and NO-generating activity were measured.

Biogel A 1.5m. The fractions containing enzyme activity were pooled and concentrated by membrane filtration (Amicon Stirred Cells, Series 8000, Beverly, MA). The proteins were loaded on a Biogel A 1.5m column (2.5 × 90 cm) pre-equilibrated with buffer A. Fractions of 6 mL were collected and assayed for protein amounts and enzyme activity. Fractions eluted from V_e/V_o (elution to void volume ratio) of 1.31 to 1.84 were collected, concentrated and used as a partially purified enzyme preparation.

Biochemical characterization of the partially purified enzyme preparation

The effects of sulfhydryl-denaturing agents on enzyme activity were examined by treating the partially purified preparation separately with NEM, IAA, pCMB or CDNB, all at 1 mM. Incubations were carried out for 15 min at 37° and the NO-generating activities of these preparations were determined as described below. The effects of sulfhydryl agents on enzyme activity were tested with various thiols at 10 μM. The effect of SDS (0.1%) on enzyme activity was examined after incubation of the preparation with the detergent for 15 min at room temperature. In separate studies, the partially purified preparation was incubated with phosphate buffer at different pHs (4 to 12), and the NO-generating activity was compared to a control sample (without proteins) at the corresponding pH.

Further enzyme purification

The partially purified preparation was used for further purification using the following columns in sequence.

Hydroxylapatite. The fractions containing enzyme activity were pooled and loaded on a 2 × 10 cm column packed with hydroxylapatite equilibrated with buffer A. The column was washed with 200 mL of buffer A and the proteins were eluted using a phosphate gradient of 0 to 200 mM sodium phosphate, pH 7.5, containing 1 mM PMSF and 0.5% CHAPS. Fractions of 2 mL were collected and used for protein and enzyme activity measurements.

Organomercurial column. Affigel 501 was first equilibrated with buffer A, and 2 mL of the resin was used to bind the proteins collected from the previous column. Binding was allowed to proceed for 2 hr at 4° and the resin was packed in a column, which was then washed with 50 mL of buffer A, followed by 20 mL of buffer A containing 1 M NaCl. The proteins were eluted with 15 mL of buffer A containing 10 mM DTT.

Biogel A 1.5m and DEAE-Sephacel repeat. The active fractions were concentrated and loaded on a Biogel A 1.5m column (1 × 50 cm). The fractions containing enzyme activity were pooled and loaded on a DEAE-Sephacel column (2 × 5 cm). The proteins were eluted using a step gradient of 20 mM NaCl (up to 200 mM final concentration in buffer A). The fractions obtained were then assayed for enzyme activity. When appropriate, protein samples were analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) by established protocols [12]. The proteins were stained either by Coomassie Blue or silver reagent (Bio-Rad).

Enzyme activity in gel slices

The separated proteins were then loaded on an SDS gel (10%) that was processed at 4°. After electrophoresis, the gel was cut into 0.5-cm slices that were incubated overnight at 4° in a buffer (10 mM Tris, pH 8, containing 0.1% SDS). The mixture was then used to determine NO-generating activity.

Determination of NO from column fractions

NO was measured in the headspace of incubation samples as previously described [1]. The protein samples were incubated with NTG (0.4 mM, unless

Table 1. Solubilization of enzyme activity by various detergents

Detergent/Buffer	Enzyme activity
Deoxycholate (2%)	4.44
Sodium cholate (2%)	4.82
Triton X-100 (1%)	3.90
Digitonin (1%)	1.54
CHAPS (3%)	7.10
Octyl glucoside (1%)	0.90
KCl (1 mM)	0.28
EGTA (5 mM)	0.1
Sucrose (250 mM)	0

Aliquots of plasma membrane were treated with various detergents or buffers as described. The supernatants obtained (0.5 mL each) after a high speed spin were directly used for measuring NO production from NTG as described. Enzyme activity is expressed as pmol of NO produced/0.1 mL of headspace at 4 hr after start of incubation.

Values represent means of duplicate measurements.

otherwise specified) in the presence of 60 μg SOD, 10 μM GSH in 50 mM phosphate buffer for various lengths of time. At different time intervals, 100 μL aliquots of headspace gas were taken out and injected onto a redox chemiluminescence detector (model 207B Sievers Research, Boulder, CO) for the quantitation of NO [1]. Calibration curves were constructed using standard NO gas.

RESULTS

Solubilization and partial purification of the NO-generating activity

Crude microsomes were treated with various detergents, and the solubilized enzyme activity responsible for producing NO from NTG was measured (Table 1). Although most detergents were able to solubilize the enzyme activity, CHAPS appeared to be the best and was subsequently used during this investigation. Neither 1 M KCl nor 5 mM EGTA solutions solubilized any enzymatic activity, suggesting that the enzyme is most likely an integral membrane protein.

Partial purification of the NO-generating activity was accomplished first by passing the solubilized microsomes through a DEAE-Sephacel column and the proteins were eluted by using a NaCl gradient (Fig. 1A). The principal enzyme activity was eluted at a NaCl concentration of about 100 mM. The fractions containing the primary enzyme activity were concentrated and the proteins then chromatographed on a Biogel A 1.5m column. The major activity peak eluted at a V_e/V_0 ratio of about 1.5, which separated quite well from the two major protein peaks (Fig. 1B). Using standard molecular weight markers, the molecular size of this protein was estimated to be about 200 kDa. Using this two-step purification process, an approximately 20-fold increase in the specific activity of the enzyme was obtained.

Properties of the partially purified enzyme preparation

Experiments were conducted to test the effects of substrate concentration, protein concentration and the length of the incubation on the NO-generating activity of the partially purified preparation. It was found that the enzyme activity increased with increasing substrate concentration; and the enzymatic reaction showed an apparent Michaelis–Menten dependency (Fig. 2). Interpretation of the kinetic constants obtained under our experimental setting is difficult since the values that could be obtained did not represent those for the actual enzymatic reaction, but instead represented the combination of the reaction and NO distribution between the headspace and the incubation mixture [1]. As expected, enzyme activity increased with increasing incubation time and protein concentration (data not shown). Maximum enzymatic activity was observed at around pH 8.0 (Fig. 3).

The presence of intracellular free thiol has been shown to be important for the pharmacological action of NTG [13, 14]. With our partially purified preparation, we found that addition of thiols such as Cys, NAC, GSH or DTT increased the NO-generating activity by 2.7- to 4.2-fold (Table 2). In comparison, no activation was observed with oxidized glutathione (GSSG), suggesting that it was the reduced state of the thiol that catalyzed the reaction. On the other hand, sulfhydryl denaturing agents such as NEM, CDNB, pCMB and IAA all inhibited NO-generating activity of the partially purified preparation by more than 80% (Table 2). The presence of a reduced sulfhydryl group in the enzyme was further supported by the fact that the enzyme activity could be trapped by passing the partially purified preparation through an organomercurial column and then be subsequently released by 10 mM DTT. This manipulation did not change the elution characteristics of the enzyme through a gel filtration column (data not shown).

Two electron donors tested, NADPH and NADH, had little apparent effect on the NO-generating activity from the microsomal preparation. Interestingly, treatment of this preparation by 0.1% SDS, which normally denatures most proteins, did not have any adverse effect on its NO-generating activity. In fact, apparent enzyme activity appeared to be enhanced in the presence of the detergent (Table 2).

Further enzyme purification

The partially purified preparation was subjected to further chromatographic purification as described in Materials and Methods, using a series of columns. Table 3 shows that each step of purification produced about 2- to 5-fold enrichment in the specific activity resulting in a cumulative enrichment of about 100-fold over CHAPS extracts.

The final protein preparation was analyzed by SDS–PAGE. A major band at about 58 kDa was observed, but two minor additional bands could be also visualized by silver staining. Since we had found earlier that the enzyme remained active even in the presence of SDS, we examined the NO-generating activities that were associated with these protein bands. We cut the gel from the SDS–PAGE into 0.5-cm slices and assayed for enzyme activity. Figure 4 shows that the major enzyme activity resided in a

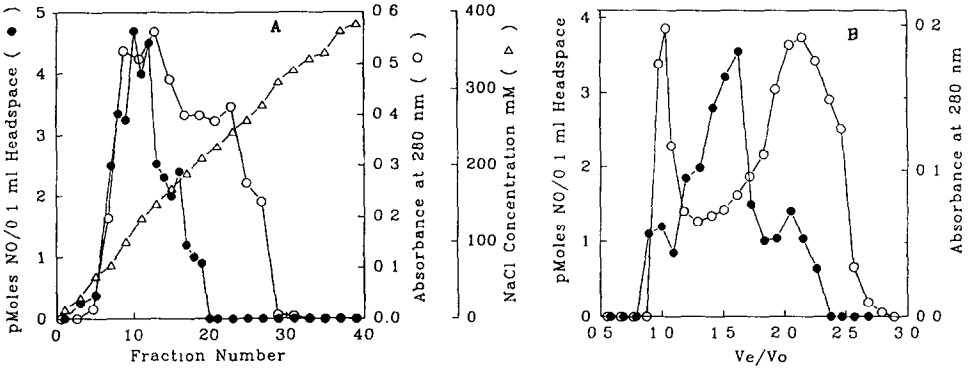

Fig. 1. NO-generating activity from NTG of CHAPS-solubilized proteins after chromatography on a DEAE-Sephacel column (panel A) and subsequently on a gel-exclusion column (panel B). The results shown are absorbance at 280 nm (○) and NO-generating activity (●), expressed as pmol of NO/0.1 mL of headspaced, in 0.5 mL of the column fractions. V_e/V_o is the elution to void volume ratio of the column.

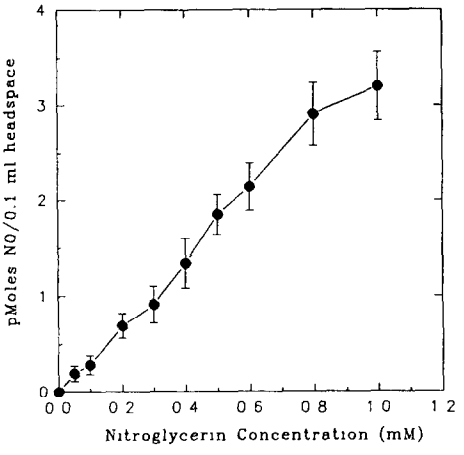

Fig. 2. Effect of substrate concentration on NO generation from the partially purified preparation.

Table 2. Effects of various reagents on NO-generating activity from NTG

Reagent (concentration)	Enzyme activity (% of control)
NEM (1 mM)	2.4 ± 2.5
pCMB (1 mM)	7.5 ± 4.5
IAA (1 mM)	18.8 ± 4.1
CDNB (1 mM)	11.9 ± 2.5
SBP (1 mM)	89.7 ± 1.3
Cys (10 μM)	353 ± 19
NAC (10 μM)	422 ± 34
GSH (10 μM)	284 ± 31
DTT (10 μM)	272 ± 33
GSSG (10 μM)	94.5 ± 16.2
NADPH (1 mM)	78.5 ± 4.7
NADH (1 mM)	79.4 ± 8.0
SDS (0.1%)	160 ± 8.0

The partially purified enzyme was incubated with the various reagents shown and assayed for the enzyme activity as described. The results shown, from triplicate measurements, are the mean ± SE percentage enzyme activities normalized with respect to that of untreated enzyme (100%). Control samples were run concurrently with each experiment.

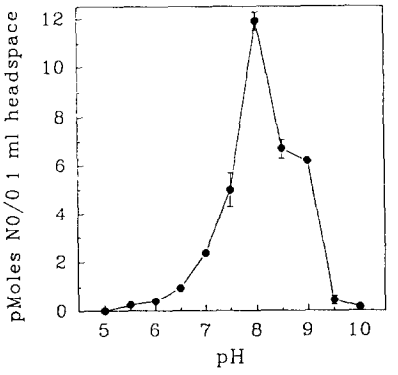

Fig. 3. Effect of pH on the NO-generating activity from NTG in the partially purified preparation. Data represent the means ± SEM of three separate experiments.

portion of the gel where the 58 kDa band was located. This purification scheme was repeated three times, with consistent results.

DISCUSSION

In this study, we showed that it is feasible to solubilize and purify an enzyme system in vascular smooth muscle cells which is responsible for producing NO from nitroglycerin. We have shown that the enzyme can be passed through four columns of different types of affinity with retention of activity, suggesting that the protein does not require another protein for its functional activity. The molecular size of the protein as estimated by gel filtration

Table 3. Enrichment of NO-generating activity after subjecting CHAPS-solubilized vascular microsomes to a series of chromatographic columns

Step	Total protein (mg)	Total activity*	Specific activity†	Fold enrichment
CHAPS extracts	291.000	34,290	120	1
DEAE-Sephacel	30.500	18,914	620	5.1
Biogel A 1.5m	6.300	12,367	1,963	16.4
Hydroxylapatite	0.960	4,495	4,316	36.0
Biogel A 1.5m repeat	0.410	3,980	9,707	80.8
DEAE-Sephacel repeat	0.290	3,284	11,325	94.3

* Each unit of enzyme activity was defined as the amount of NO produced in total headspace after 4 hr of incubation.
† Total activity/mg protein.

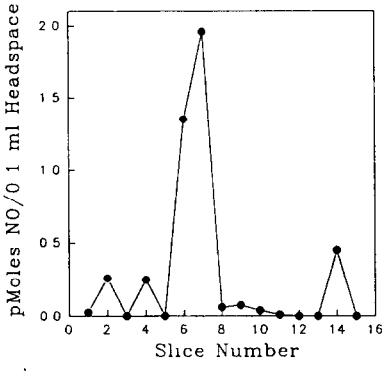

Fig. 4. NO-generating activity from NTG from SDS–PAGE gel slices after the last step of purification shown in Table 3.

chromatography was about 200 kDa, which is consistent with a previous estimation determined by the technique of radiation inactivation [4]. We have shown in other reports that this enzyme appears to convert other organic nitrates such as pentaerythrityl trinitrate and isosorbide dinitrate to NO [3], but is different from the enzyme responsible for the conversion of organic nitrites to NO [5].

The partially purified preparation exhibited biochemical properties typical of enzymes. The NO-generating activity increased with increasing substrate concentration (in an apparent Michaelis–Menten manner), increasing protein concentration and time of incubation. We have shown previously, with an unpurified microsomal preparation, that Michaelis–Menten kinetics were clearly observed, with an apparent K_m of 285 μM [4]. In the partially purified preparation, the apparent K_m appeared to be considerably higher, and complete Michaelis–Menten behavior was not fully characterized.

An important finding is that the enzyme appears to contain a free sulfhydryl group which is required for enzymatic activity. This conclusion is based on the observation that sulfhydryl donating agents enhanced enzyme activity, whereas sulfhydryl denaturing compounds had the opposite effect (Table 2). In addition, we showed that the enzyme activity could bind with an organomercurial column, but this binding could be reversed by a reducing agent, DTT. This result suggested that the enzyme contains a free -SH group that can undergo reversible disulfide bond formation. Interestingly, the pH optimum of the enzyme activity was found to be around 8.0, close to the pK_a value of the -SH group of cysteine, 8.33 [15]. It is conceivable, therefore, that a pH-dependent proteination and deproteination of the -SH group resident in the enzyme could be part of the mechanism of enzyme action.

It has been proposed that vascular tolerance toward nitrates may have been brought about by depletion of critical intracellular sulfhydryl groups, which are necessary to mediate nitrate action [13, 14]. Interestingly, it has been observed that maximal tolerance occurs prior to a detectable decrease in tissue sulfhydryl content [16]. Our finding that the nitrate metabolizing enzyme contains a free sulfhydryl function could possibly reconcile these apparently contradictory observations since oxidation of the thiol on the enzyme (as a result of chronic nitrate exposure) would not be easily detectable when total tissue sulfhydryl content was measured.

The mechanism of thiol-activation of enzyme activity may not be simply due to the availability of an electron source, since at least two other electron donors, NADH and NADPH, which are known to participate in the electron transport chain, failed to increase enzyme activity. It is conceivable, therefore, that thiol groups could participate as a specific co-factor for the reaction.

Another interesting property of the enzyme is that the NO-generating activity functions even in the presence of an ionic detergent, SDS. Since SDS is known to disrupt most of the intra- and inter-molecular forces involving non-covalent interactions in proteins, this finding suggests that perhaps the primary structure of the protein, rather than its native conformation, may be sufficient for enzymatic function. Another explanation for this observation is that SDS may be able to denature an inhibitor of the nitrate metabolizing enzyme.

Several recent reports suggested that a cytochrome P450 enzyme may be responsible for converting nitrates to NO [8–11]. A number of observations in the present study indicated, however, that the microsomal enzyme we have examined is not a cytochrome P450 enzyme. These observations include: the molecular size of the enzyme, its stability towards detergents such as SDS, its apparent need for thiol, and the lack of dependency on NADH or NADPH. We have documented previously that this microsomal enzyme is also not a glutathione-*S*-transferase [4].

The active subunit of the enzyme has a molecular size of about 58 kDa. Since the molecular size of the native enzyme is about 200 kDa, we propose that this enzyme exists as a tetramer in the cell membrane. At this point, because of a number of factors, viz. the poor yield of the enzyme, lack of any affinity chromatography step, and the difficulty in obtaining a large amount of smooth muscle cells from the coronary artery, it has not yet been possible to subject the purified protein to further biochemical analyses such as amino acid composition and amino acid sequence. Further work is necessary to define the exact nature of this enzyme at a molecular level.

Acknowledgements—This work was supported in part by NIH Grant HL22273. We are thankful to Shawn Doran, Sun Mi Fung and David Soda for their technical assistance.

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