Purification and Reconstitution of Functional Shaker K^+ Channels Assayed with a Light-Driven Voltage-Control System†

Lucia Santacruz-Toloza, Eduardo Perozo,‡ and Diane M. Papazian*

Department of Physiology (School of Medicine), Molecular Biology Institute, and Jules Stein Eye Institute, University of California at Los Angeles, Los Angeles, California 90024-1751

Received December 2, 1993®

ABSTRACT: Voltage-dependent potassium channels are integral membrane proteins that control the excitability of nerve and muscle. The cloning of genes for K^+ channels has led to structure/function analysis using a combination of site-directed mutagenesis and electrophysiology. As a result, much has been learned about how these proteins work. A deeper understanding of their function will require detailed structural characterization, however. We now report the purification of Shaker K^+ channels from an insect expression system using immunoaffinity methods. The purified channels have been reconstituted, assayed using a novel, light-driven, vesicular voltage-control system, and shown to be functional. This approach will enable us to compare and optimize methods for protein production and purification. Purification of active protein is a prerequisite for detailed structural analysis, since activity is the key indication that the structural integrity of the channel has been preserved during biochemical procedures. Thus, this work represents a first step toward the determination of the structure of Shaker K^+ channels.

The gene encoding the voltage-dependent Shaker K^+ channel was cloned from the fruit fly, *Drosophila melanogaster*, using a genetic approach (Papazian et al., 1987; Kamb et al., 1987; Pongs et al., 1988). This approach was necessary because K^+ channel proteins had not been purified, and antibodies and partial protein sequences needed for other cloning strategies were not available. Subsequently, genes for other K^+ channels were cloned by homology and expression methods (Tempel et al., 1988; Takumi et al., 1988; Butler et al., 1989; Frech et al., 1989). Using a combined approach of site-directed mutagenesis and electrophysiological assay, much has been learned about the contributions of different regions of the sequence to the functional properties of K^+ channels, including voltage-dependent activation (Papazian et al., 1991; Liman et al., 1991; Logothetis et al., 1992), K^+ permeation and selectivity (Yellen et al., 1991; Hartmann et al., 1991; Yool & Schwarz, 1991), and inactivation (Hoshi et al., 1990; Zagotta et al., 1990). A key limitation in our understanding of K^+ channel mechanisms, however, is the lack of detailed structural information (Miller, 1991). To overcome this deficit, a pure, structurally-intact preparation of K^+ channel protein is required.

A recombinant baculovirus encoding the Shaker H4 K^+ channel, expressed in Sf9 cells in culture, produces a large amount of Shaker protein (Klaiber et al., 1990). Patch clamp analysis has shown that some of this protein can be detected as active channels on the cell surface (Klaiber et al., 1990). However, immunofluorescence experiments indicate that a large fraction of the Shaker protein remains intracellular, where its functional competence is unknown (unpublished observations).

Sf9 cells infected with the recombinant baculovirus are useful as a source of Shaker channels for purification for three main reasons. First, this system makes a large amount of Shaker protein, at least some of which has been shown to be functional (Klaiber et al., 1990). In contrast, in natural sources, the protein is present in low amounts. Second, multiple genes that encode similar K^+ channel proteins are expressed in many natural sources (Stuhmer et al., 1989; Wei et al., 1990; Swanson et al., 1990), raising the possibility of purifying a mixture of different channels, as is the case for K^+ channels purified from brain tissue (Rehm et al., 1989). In an expression system that lacks endogenous voltage-dependent K^+ channels, this problem is avoided. Third, K^+ channels are thought to be tetramers (MacKinnon, 1991) that may consist of identical or nonidentical subunits (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990; Sheng et al., 1993; Wang et al., 1993). In the baculovirus system, the channel is expected to be formed by identical subunits since only one Shaker K^+ channel sequence is being expressed. Purification of a homogeneous channel preparation with a defined subunit composition will be an asset for structural studies. We now report that Shaker K^+ channel protein has been purified to homogeneity from Sf9 cells infected with a recombinant baculovirus. The purified channels have been reconstituted and shown to be functionally active using a light-driven, vesicular voltage-control system (Perozo & Hubbell, 1993).

MATERIALS AND METHODS

Materials. Sf9 cells were obtained from the American Type Culture Collection and were grown and infected as described (Klaiber et al., 1990). Wild-type and recombinant baculoviruses were kindly provided by Dr. Chris Miller (Brandeis University). Anti-Shaker antibodies were produced by Berkeley Antibody Co. (Richmond, CA) and were the generous gift of Dr. Lily Jan (University of California, San Francisco). Shaker peptide was synthesized by the UCLA Peptide Facility. Bacteriorhodopsin was kindly provided by Dr. Roberto Bogomolni (University of California, Santa Cruz). Other reagents were obtained from the following suppliers: protease inhibitors, Boehringer Mannheim; lipids, Avanti Polar Lipids;

† Supported by grants from the NIH (GM43459), the Esther A. and Joseph Klingenstein Fund, and the Pew Charitable Trusts (E.P. and D.M.P.).

* Address correspondence to this author at the Department of Physiology, UCLA School of Medicine, Los Angeles, California 90024-1751. Phone: (310) 206-7043. Fax: (310) 206-5661.

‡ Present address: Centro de Bioquimica y Biofisica, Instituto Venezolano de Investigaciones Cientificas, Apartado Postal 21827, Caracas 1020A, Venezuela.

® Abstract published in *Advance ACS Abstracts,* January 15, 1994.

0006-2960/94/0433-1295$04.50/0

Lubrol PX and protein A–Sepharose beads, Sigma; dimethyl pimelimidate dihydrochloride, Pierce; ^{86}RbCl, Amersham Life Science; Dowex 50W ×8, Bio-Rad.

Membrane Fraction Isolation, Solubilization, and Reconstitution. Sf9 cells were infected with the recombinant (Shaker H4) or wild-type baculoviruses at a multiplicity of 10. At 48-h post-infection, cells were resuspended in 10% w/v sucrose prepared in 150 mM KCl, 10 mM magnesium acetate, and 20 mM Hepes, pH 7.5, supplemented with 40 μg/mL bestatin, 50 μg/mL antipain, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, 2 μg/mL aprotinin, 25 μg/mL 4-(amidinophenyl)methanesulfonyl fluoride, 0.5 mM phenylmethanesulfonyl fluoride, 0.1 mM EDTA, and 5 mM dithiothreitol. Cells were homogenized in a Dounce homogenizer (30–40 strokes) followed by freezing in liquid N_2 and brief sonicating. The cell homogenate was centrifuged at 170000*g* for 30 min at 4 °C on a 10%/20%/50% sucrose gradient prepared in the same buffer lacking bestatin and antipain. A membrane fraction was collected from the 20–50% interface, diluted at least 2.5-fold in 150 mM KCl/20 mM Hepes, pH 7.5, and pelleted by centrifugation at 170000*g* for 10 min at 4 °C. Membrane proteins were solubilized in 150 mM KCl, 20 mM Hepes, pH 7.5, 2% (w/v) Lubrol PX, and 2 mg/mL egg phosphatidylcholine, supplemented with protease inhibitors as above. Insoluble material was removed by centrifugation at 100000*g* for 30 min at 4 °C. The solubilized sample was supplemented with an equal volume of reconstitution buffer: 150 mM KCl, 20 mM Hepes, pH 7.5, 4% w/v Lubrol PX, and 40 mg/mL egg phosphatidylcholine, supplemented with protease inhibitors. The preparation was dialyzed at 4 °C against 200 mM *N*-methylglucamine titrated with H_2SO_4 to pH 7.5 and 2.5 mM dithiothreitol, and then treated with 0.75 g/mL Biobeads (Bio-rad) for 4–6 h at 4 °C.

Co-Reconstitution and Functional Assays. Purple membranes from *Halobacterium halobium* were isolated on a sucrose density gradient (Oesterhelt & Stoeckenius, 1974), and bacteriorhodopsin (bR) was reconstituted by the reverse-phase evaporation method (Rigaud et al., 1983; Szoka & Papahadjopoulos, 1978). Bacteriorhodopsin-containing proteoliposomes (20 mg/mL egg phosphatidylcholine, 0.6 mg/mL bR) in 200 mM *N*-methylglucamine sulfate, pH 7.5, were fused with Shaker proteoliposomes (20 mg/mL egg phosphatidylcholine, 0.3 mg/mL solubilized membrane fraction or 5 μg/mL purified protein) in a 3:1 ratio by 5 freeze/thaw cycles followed by sonication for 15 s (Perozo & Hubbell, 1993). Flux assays were conducted at 20 °C. A negative bias (holding) potential was generated by a 10-fold dilution of the fused vesicles with 200 mM *N*-methylglucamine–nitrate, pH 7.5, and incubation for 1 h in the dark. At time = 0, this preparation (100 μL) was mixed with 0.5–5 μCi of $^{86}Rb^+$ in the dark. Where indicated, vesicles were illuminated continuously starting at 1 min with a halogen lamp (150 mW) equipped with a heat filter. Samples (30 μL) were taken at 0.5, 1.5, and 2.5 min and immediately applied to a Dowex 50W ×8 ion-exchange column to remove unincorporated $^{86}Rb^+$. Dowex columns, prepared as described (Papazian et al., 1979), were eluted with 1 mL of 300 mM glucose, 10 mM Tris, and 2 mM $MgSO_4$, pH 7.3, under gravity flow. The eluant was subjected to scintillation counting. Where indicated, $Ba(NO_3)_2$ was added to a final concentration of 2 mM to the vesicle suspension in *N*-methylglucamine–nitrate. Vesicles were incubated in $Ba(NO_3)_2$ for 10 min in the dark before addition of the radioisotope. Transmembrane potentials of the reconstituted vesicles were determined using TPP^+ (tetraphenylphosphonium ion) electrodes (Kamo et al., 1979). The response of the electrodes followed Nernst's law up to 10^{-6} M TPP^+.

Purification of Shaker K^+ Channels. To purify Shaker K^+ channels, a membrane fraction was prepared and solubilized, applied to fresh immunoaffinity resin (previously resuspended in solubilization buffer), and incubated for 16 h at 4 °C with gentle agitation. The unbound material was collected, and the resin was extensively washed with solubilization buffer. A 1 mM solution of the elution peptide prepared in the same buffer was applied to the resin, and the suspension was incubated for 22 h at 20 °C with gentle agitation. The eluted fraction was collected and supplemented with an equal volume of reconstitution buffer. The sample was then dialyzed, reconstituted, and assayed for $^{86}Rb^+$ uptake. The immunoaffinity resin was prepared as follows: 350 μL (0.9 mg of IgG) of rabbit serum raised against the synthetic peptide EEEDTLNLPKAPVSPQDKS was incubated with 0.5 g of protein A–Sepharose beads (preequilibrated in phosphate-buffered saline, PBS, pH 7.2) for 1.5 h at 20 °C. The resin was extensively washed with PBS and then equilibrated in 200 mM sodium borate, pH 9.0. IgG molecules were cross-linked to protein A by reaction with 20 mM dimethyl pimelimidate dihydrochloride in borate buffer. The unreacted cross-linker was inactivated by incubating the resin in 200 mM Tris, pH 8.5, for 2 h at 20 °C. The resin was subjected to a series of low- and high-pH washes using 200 mM glycine buffer (pH 2.5) and 200 mM sodium monophosphate (pH 12.5), alternating with phosphate buffer washes.

Electrophoresis and Immunoblots. For electrophoresis and immunoblot analysis, samples of the various fractions obtained during the purification procedure were diluted with electrophoresis sample buffer (Laemmli, 1970) containing 10% v/v 2-mercaptoethanol. Gels were silver stained twice using Bio-Rad's silver stain kit, following the manufacturer's protocol. Immunoblots were performed as previously described (Schwarz et al., 1990).

Protein Quantification. Total protein in the membrane and solubilized fractions was quantified using the Lowry protein assay (Lowry et al., 1951). Purified samples were quantified by densitometry after electrophoresis and silver staining. A calibration curve was obtained simultaneously by running a set of bovine serum albumin (BSA) concentrations. The absolute intensities were fit with an equation of the form: $OD = 1/\{1 + \exp[(C_{BSA} - C_{BSA,1/2})/k]\}$, where $C_{BSA,1/2}$ is the BSA concentration that produces half of the maximum optical density and k is a slope factor.

RESULTS

To monitor the purification of active Shaker K^+ channels, a functional assay is required. We were interested in determining the activity of the bulk population of channels rather than single channels because a bulk flux assay makes it feasible to compare and optimize biochemical protocols. We have assayed Shaker K^+ channel activity in reconstituted vesicles using a voltage-control system driven by bacteriorhodopsin (Perozo & Hubbell, 1993), a light-activated proton pump (Figure 1). This system has been used previously to activate pharmacologically-unmodified Na^+ channels purified from eel electroplax (Perozo & Hubbell, 1993). Bacteriorhodopsin (bR) was reconstituted in phosphatidylcholine vesicles using reverse-phase evaporation (Perozo & Hubbell, 1993; Rigaud et al., 1983), a method that yields approximately 95% of the protein oriented inside-out. Reconstituted vesicles containing Shaker protein were fused with bR vesicles by

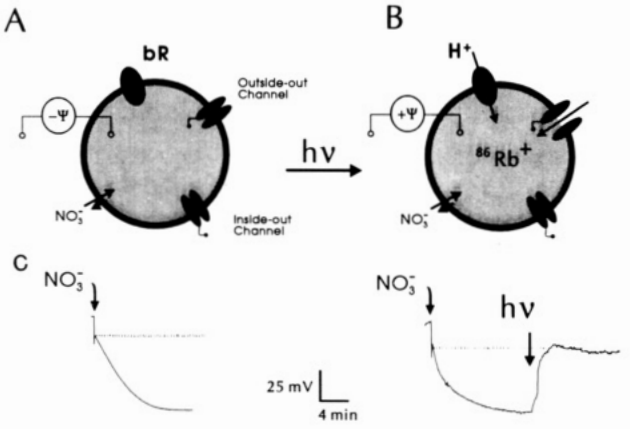

FIGURE 1: Schematic diagram of the light-driven vesicular voltage-control system. The system is generated after fusion of vesicles containing inside-out bacteriorhodopsin and vesicles containing Shaker channels (presumably oriented in a 50% inside-out configuration). (A) An idealized vesicle in the dark is shown. (B) Membrane depolarization occurs after light-induced proton pumping. Outside-out Shaker channels open to allow $^{86}Rb^+$ influx, whereas inside-out, inactivated, Shaker channels remain closed. (C) The transmembrane potential of vesicles, $\Delta\Psi$, can be monitored as a function of time using TPP^+ electrodes (Kamo et al., 1979). The permeant anion, nitrate, generates a hyperpolarized bias potential in the presence of an impermeable counter-cation (left). Illumination of bR is capable of reverting the bias potential to at least 0 mV (and probably more positive) at the highest light intensities (150 mW) (right). The dotted line represents $\Delta\Psi = 0$, and is the result of the instantaneous dilution of TPP^+. The changes in $\Delta\Psi$ of the reconstituted vesicles can also be measured independently using the spin-labeled phosphonium (Perozo & Hubbell, 1993).

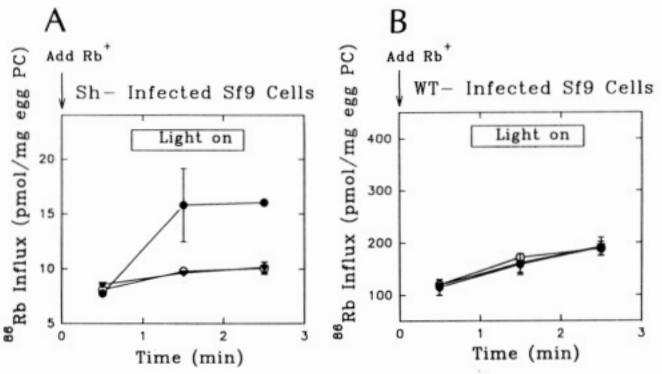

FIGURE 2: Functional assay of reconstituted membrane preparations from Sf9 cells infected with the Shaker recombinant virus or a wild-type baculovirus. A membrane fraction was isolated, and membrane proteins were solubilized and reconstituted into phosphatidylcholine vesicles. These vesicles were then fused with bR-containing vesicles and incubated at 20 °C in a nitrate-containing solution. At time = 0, $^{86}Rb^+$ was added; samples were collected 0.5, 1.5, and 2.5 min later. Intravesicular isotope was quantified by scintillation counting and plotted as a function of time. Filled symbols: samples were continuously illuminated from 1 min on. Open symbols: samples were kept in darkness. Triangles: the K^+ channel blocker Ba^{2+} was added to a final concentration of 2 mM, 10 min before the addition of $^{86}Rb^+$. Circles: no Ba^{2+} was added. Each point is the average of three determinations; error bars represent the standard deviation of the mean. (A) Reconstituted preparation from cells expressing Shaker K^+ channels showed a light-dependent uptake of $^{86}Rb^+$ that was blocked by external Ba^{2+}. This uptake was not observed in a reconstituted preparation from cells infected with a wild-type baculovirus (B).

freeze/thaw/sonication. A diffusion potential, inside negative, was established by incubating the fused vesicles in a solution containing the permeant anion nitrate and an impermeant counter-cation, *N*-methylglucamine. This hyperpolarized potential was necessary to remove fast and slow channel inactivation (Hoshi et al., 1991) that accumulates after the Sf9 cells are disrupted and the Shaker protein is solubilized. Subsequently, the vesicles were incubated in the presence of $^{86}Rb^+$ and illuminated. Light activates bR to pump protons into the vesicles, rapidly depolarizing them (Figure 1B). If the vesicles contain active, properly oriented (outside-out) Shaker channels, they should open, allowing influx of $^{86}Rb^+$. (Channels reconstituted inside-out will not be activated by this procedure, for they are still inactivated.) Subsequently, the open channels will inactivate, trapping any accumulated tracer inside the vesicles. After allowing time for the depolarization-induced uptake of $^{86}Rb^+$ into the vesicles, the unincorporated tracer was rapidly removed by ion-exchange chromatography. Characterization of this system indicates that the initial hyperpolarization achieved by incubation with nitrate is approximately −70 mV and that the rate and extent of depolarization depend on the intensity of the illumination (Perozo & Hubbell, 1993).

This assay can unambiguously identify light-dependent (voltage-activated) $^{86}Rb^+$ flux through Shaker channels (Figure 2). Membrane fractions were isolated from Sf9 cells that had been infected either with the recombinant baculovirus encoding Shaker H4 or with a wild-type baculovirus. Membrane proteins were solubilized with the detergent Lubrol-PX, dialyzed, and reconstituted in phosphatidylcholine vesicles by treatment with Biobeads. A light-dependent uptake of $^{86}Rb^+$, blockable by external Ba^{2+}, a K^+ channel blocker (Eaton & Brodwick, 1980), was observed in the preparation derived from Shaker-infected Sf9 cells (Figure 2A). $^{86}Rb^+$ influx was complete by the first time point after illumination of the sample, consistent with the extremely rapid rates of ion flux and inactivation of Shaker channels. In contrast, light did not stimulate $^{86}Rb^+$ uptake into vesicles containing proteins from Sf9 cells infected with a wild-type baculovirus (Figure 2B). Similar results were obtained after solubilizing with the detergent CHAPS (3–[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (data not shown).

A membrane fraction from Sf9 cells infected with the recombinant Shaker baculovirus was solubilized with Lubrol and applied to an immunoaffinity resin made from an antipeptide antibody (directed against the putative second extracellular loop of the protein) chemically cross-linked to protein A–Sepharose beads. After solubilized Shaker protein bound to the resin, other proteins in the preparation were removed by extensive washes. Then, bound protein was eluted in a batch treatment using the synthetic peptide against which the antiserum was directed. The purification was monitored by SDS–polyacrylamide gel electrophoresis, immunoblotting, and protein assay (Figure 3A,B, Table 1). According to silver staining of protein gels (Figure 3A), the preparation eluted from the immunoaffinity resin was homogeneous and contained bands that were identified as Shaker protein on parallel immunoblots (Figure 3B). Shaker protein made in Sf9 cells exists in three forms differing in the extent of glycosylation (unpublished observations). This accounts for the appearance of a triplet of protein bands in the purified preparation on gels and immunoblots. From a 10-mL starting culture, approximately 10 μg of purified protein was obtained. We estimate that the Shaker protein has been enriched about 400-fold by the purification procedure (Table 1).

The purified Shaker protein was reconstituted and assayed for voltage-dependent, Ba^{2+}-inhibitable, $^{86}Rb^+$ uptake using the light-driven, vesicular voltage-control system. Figure 3C shows that the purified channel was active. We have observed some variability in the level of activity among different preparations of purified protein, however, We have not

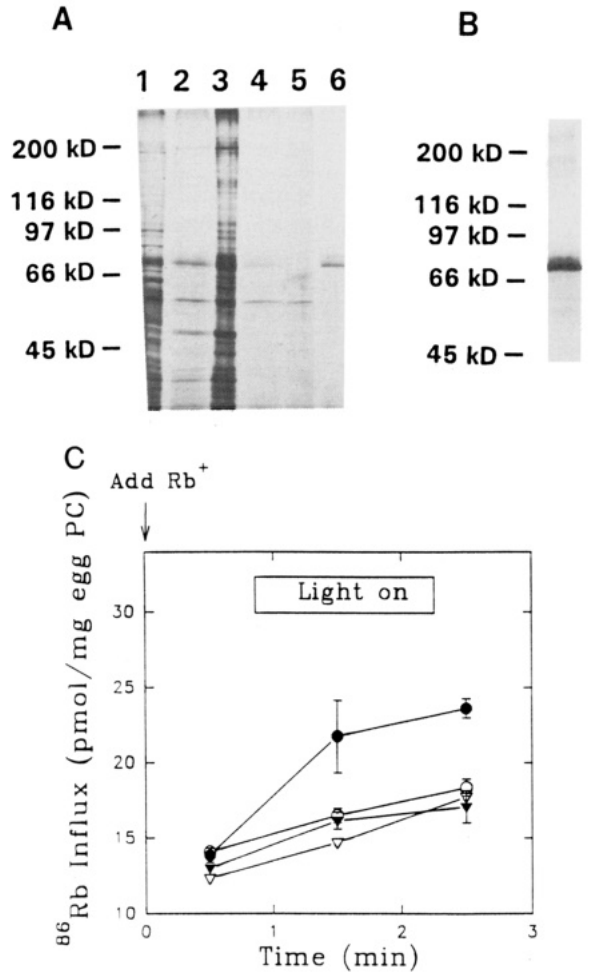

FIGURE 3: Purification and assay of Shaker K^+ channels. (A) Silver-stained polyacrylamide gel. Protein samples collected at various stages of the purification were subjected to electrophoresis on a 7.5% polyacrylamide gel/4% stack. Samples are as follows: lane 1, cell homogenate; lane 2, membrane fraction, lane 3, insoluble material; lane 4, solubilized preparation; lane 5, protein that did not bind to the immunoaffinity resin; lane 6, protein eluted from resin with peptide. The lanes did not contain equal amounts of protein. (B) Immunoblot of purified protein eluted from the resin with peptide, probed with an anti-Shaker antibody (Schwarz et al., 1990). The blot indicates that the purified protein can be identified immunologically as Shaker protein. (C) Functional assay of purified, reconstituted preparation. Symbols are as defined in Figure 2.

Table 1: Protein Recovery during Shaker Protein Purification[a]

step	total protein (mg)	enrichment factor
total membrane fraction	2.4	
solubilized protein	1.16	2.8
after elution	0.01	280
after reconstitution	0.007	400

[a] Purification started with 10 mL of culture containing about 2×10^6 cells/mL. The enrichment factor does not take into account loss of Shaker protein during the purification.

determined what fraction of the protein in our purified preparation is active. To estimate this, more detailed characterization of the reconstituted system will be necessary. For example, only outside-out Shaker channels co-reconstituted with inside-out bR can open in response to the light-induced depolarization. The fraction of reconstituted vesicles containing this configuration has not yet been determined.

DISCUSSION

The light-driven vesicular voltage clamp will be useful for comparing and optimizing different protocols of protein production and purification. This functional assay is easy enough for routine use and gives information about the activity of the bulk population of channels. With additional characterization, this approach should enable us to determine what fraction of the protein population is active and able to undergo the voltage-dependent conformational changes that lead to opening. This feature of the light-driven voltage-control system is not shared by other commonly-used approaches for studying purified channel proteins. Although other assays, such as planar bilayer reconstitution (Labarca & Latorre, 1992) or liposome patch clamp (Correa & Agnew, 1988), give detailed information about the electrophysiological properties of individual channels, these methods are able to assay only a tiny fraction of the molecules in the preparation and do not reveal the overall activity of the protein in the preparation. Another common approach, the binding of high-affinity ligands, such as toxins, to solubilized channel protein, does not directly measure activity.

The purification of active Shaker K channels represents a first step toward obtaining detailed structural information, which is critically needed as a framework for structure/function analysis (Miller, 1991). Demonstration of channel activity prior to structural studies is essential since activity is the primary indication that the structural integrity of the channel has been preserved during biochemical manipulations. However, only a small amount of protein has been purified, compared to the amounts needed for structural determination using crystallographic, spectroscopic, or microscopic techniques. To date, Shaker protein has been purified from small cultures of infected cells; improvements in the yield could be achieved by producing protein on a larger scale than we have attempted so far. In addition, alternative expression systems and purification strategies can now be compared to optimize the yield of active channel protein.

ACKNOWLEDGMENT

We thank Drs. W. L. Hubbell for illuminating discussions, Lily Jan for anti-Shaker antibodies, Chris Miller for baculoviruses, Roberto Bogomolni for bacteriorhodopsin, and Janis Young for peptide synthesis.

REFERENCES

Butler, A., Wei, A., Baker, K., & Salkoff, L. (1989) *Science 243*, 943–947.

Christie, M. J., North, R. A., Osborne, P. B., Douglass, J., & Adelman, J. P. (1990) *Neuron 2*, 405–411.

Correa, A. M., & Agnew, W. S. (1988) *Biophys. J. 54*, 569–575.

Eaton, D. C., & Brodwick, M. C. (1980) *J. Gen. Physiol. 75*, 727–750.

Frech, G. C., VanDongen, A. M. J., Schuster, G., Brown, A. M., & Joho, R. H. (1989) *Nature 340*, 642–645.

Hartmann, H. A., Kirch, G. E., Drewe, J. A., Taglialatela, M., Joho, R., & Brown, A. M. (1991) *Science 251*, 942–944.

Hoshi, T., Zagotta, W. N., & Aldrich, R. W. (1990) *Science 250*, 533–538.

Hoshi, T., Zagotta, W. N., & Aldrich, R. W. (1991) *Neuron 7*, 547–556.

Isacoff, E. Y., Jan, Y. N., & Jan, L. Y. (1990) *Nature 345*, 530–534.

Kamb, A., Iverson, L. E., & Tanouye, M. A. (1987) *Cell 50*, 405–413.

Kamo, N., Muratsugu, M., Hongoh, R., & Kobatake, Y. (1979) *J. Membr. Biol. 49*, 105–121.

Klaiber, K., Williams, N., Roberts, T. M., Papazian, D. M., Jan, L. Y., & Miller, C. (1990) *Neuron 5*, 221–226.

Labarca, P., & Latorre, R. (1992) *Methods Enzymol. 207*, 447–463.

Laemmli, U. K. (1970) *Nature 227*, 680–685.
Liman, E. R., Hess, P., Weaver, F., & Koren, G. (1991) *Nature 353*, 752–756.
Logothetis, D. E., Movahedi, S., Satler, C., Lindpaintner, K., & Nadal-Ginard, B. (1992) *Neuron 8*, 531–540.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–269.
MacKinnon, R. (1991) *Nature 350*, 232–235.
Miller, C. (1991) *Science 252*, 1092–1096.
Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667–678.
Papazian, D. M., Rahamimoff, H., & Goldin, S. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3708–3712.
Papazian, D. M., Schwarz, T. L., Tempel, B. L., Jan, Y. N., & Jan, L. Y. (1987) *Science 237*, 749–753.
Papazian, D. M., Timpe, L. C., Jan, Y. N., & Jan, L. Y. (1991) *Nature 349*, 305–310.
Perozo, E., & Hubbell, W. L. (1993) *Biochemistry 32*, 10471–10478.
Pongs, O., Kecskemethy, N., Muller, R., Krah-Jentgens, I., Baumann, A., Klitz, H. H., Canal, I., Llamazares, S., & Ferrus, A. (1988) *EMBO J. 7*, 1087–1096.
Canal, I., Llamazares, S., & Ferrus, A. (1988) *EMBO J. 7*, 1087–1096.
Rehm, H., Pelzer, S., Cochet, C., Chambaz, E., Tempel, B. L., Trautwein, W., Pelzer, D., & Lazdunski, M. (1989) *Biochemistry 28*, 6455–6460.
Rigaud, J. L., Bluzat, A., & Buschlen, S. (1983) *Biochem. Biophys. Res. Commun. 111*, 373–382.
Ruppersberg, J. P., Schroter, K. H., Sakmann, B., Stocker, M., Sewing, S., & Pongs, O. (1990) *Nature 345*, 535–537.
Schwarz, T. L., Papazian, D. M., Carretto, R. C., Jan, Y. N., & Jan, L. Y. (1990) *Neuron 2*, 119–127.
Sheng, M., Liao, Y. J., Jan, Y. N., & Jan, L. Y. (1993) *Nature 365*, 72–75.
Stuhmer, W., Ruppersberg, J. P., Schroter, K. H., Sakmann, B., Stocker, M., Giese, K. P., Perschke, A., Baumann, A., & Pongs, O. (1989) *EMBO J. 8*, 3235–3244.
Swanson, R., Marshall, J., Smith, J. S., Williams, J. B., Boyle, M. B., Folander, K., Luneau, C. J., Antanavage, J., Oliva, C., Buhrow, S. A., Bennett, C., Stein, R. B., & Kaczmarek, M. K. (1990) *Neuron 4*, 929–939.
Szoka, F., & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4194–4198.
Takumi, T., Ohkubo, H., & Nakanishi, S. (1988) *Science 242*, 1042–1045.
Tempel, B. L., Jan, Y. N., & Jan, L. Y. (1988) *Nature 332*, 837–839.
Wang, H., Kunkel, D. D., Martin, T. M., Schwartzkroin, P. A., & Tempel, B. L. (1993) *Nature 365*, 75–79.
Wei, A., Covarrubias, M., Butler, A., Baker, K., Pak, M., & Salkoff, L. (1990) *Science 248*, 599–603.
Yellen, G., Jurman, M. E., Abramson, T., & MacKinnon, R. (1991) *Science 251*, 939–941.
Yool, A. J., & Schwarz, T. L. (1991) *Nature 349*, 700–704.
Zagotta, W. N., Hoshi, T., & Aldrich, R. W. (1990) *Science 250*, 568–571.