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# Bacterial Synthesis, Purification, and Solubilization of Membrane Protein KCNE3, a Regulator of Voltage-Gated Potassium Channels

**S. A. Goncharuk*, A. A. Shulga, Ya. S. Ermolyuk, P. K. Kuzmichev, V. A. Sobol, E. V. Bocharov, V. V. Chupin, A. S. Arseniev, and M. P. Kirpichnikov**

*Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117997 Moscow, Russia; fax: (495) 335-5033; E-mail: goncharuk@nmr.ru*

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**Abstract**—An efficient method is described for production of membrane protein KCNE3 and its isotope labeled derivatives ($^{15}$N-, $^{15}$N-/$^{13}$C-) in amounts sufficient for structural-functional investigations. The purified protein preparation within different detergent micelles was characterized using dynamic light scattering, CD spectroscopy, and NMR spectroscopy. It is shown that within DPC/LDAO micelles the protein is in monomeric form and acquires mainly α-helical conformation. The existence of cross-peaks for all glycines of the $^{15}$N-HSQC NMR spectra as well as relatively small line widths (~20 Hz) confirm the high quality of the preparation and the possibility of obtaining structural-dynamic information on KCNE3 by high resolution heteronuclear NMR spectroscopy.

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*Key words*: KCNE (MiRP), membrane protein, expression, purification, dynamic light scattering, NMR

The integral membrane protein KCNE3 (MiRP2) is a member of the KCNE family of regulators of voltage-gated potassium channels that influence the ion conductivity and selectivity, ion flux rate, drug sensitivity, as well as some other channel properties [1-4]. The KNCE3 protein consists of 103 amino acid residues and has one transmembrane region. Mutations in KCNE3 are associated with different diseases such as familial periodic paralysis or atrial fibrillation [5-9]. There are a number of works on functional investigation of KCNE3. It has been shown that in experimental systems (Chinese hamster ovary cells, *Xenopus* oocytes) the protein forms stable complexes with potassium channels Kv3.4, HERG, and KCNQ1 by changing their biophysical properties [10-12]. However, the structural features that form the basis of KCNE3 functioning are not studied yet.

In this work, an efficient method of obtaining KCNE3 and its isotope labeled derivatives is described.

## MATERIALS AND METHODS

*Escherichia coli* strains XL-10 and BL21(DE3)pLysS (Stratagene, USA), plasmids pGEMEX-1 (Novagen, USA) and pGEMEX-1/Thio, and chromatographic resins Chelating Sepharose FF (GE Healthcare, USA) and C4 Diasorb (Biokhimmak ST, Russia) were used in this work. Oligonucleotides were synthesized by Evrogen Company (Institute of Bioorganic Chemistry, Russian Academy of Sciences). DNA was sequenced by the Interinstitute Center of Joint Use "GENOME" (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences). Reagents of CIL (USA) $^{15}NH_4Cl$ and $^{13}C_6H_{12}O_6$ were used for $^{15}$N and $^{13}$C introduction.

**Cloning and expression of *KCNE3* gene.** The *KCNE3* gene was assembled using two successive PCR stages from ten chemically synthesized oligonucleotides partially overlapped along its sequence. The oligonucleotide

*Abbreviations*: DPC, dodecyl phosphocholine; H6, histidine tag (HHHHHH); HSQC, Heteronuclear Single Quantum Correlation; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl β-D-thiogalactoside; GS, flexible linker (GSGSG); LDAO, lauryl dimethylaminoxide; LMPC, lysomyristoyl phosphatidylcholine; LMPG, lysomyristoyl phosphatidylglycerol; LPPG, lysopalmitoyl phosphatidylglycerol; TR, thrombin recognition site (LVPRGS).

* To whom correspondence should be addressed.

lengths were from 45 to 49 nucleotides. A sequence of 18 nucleotides encoding the thrombin recognition site was introduced into the 5′-terminal primer. This sequence was used for recombination by PCR technique of *Thio-TR* (containing the gene of thioredoxin) and *KCNE3* genes. The *Hin*dIII restriction site was introduced into the 3′-terminal primer. To obtain the *Thio-TR* fragment, PCR was carried out using forward (TAATACGACTCACTATAGGG) and reverse (GGATCCACGAGGAACCAGACCAGAACCAGAGCCGTGAT) primers. The *Thio* nucleotide sequence within vector pGEMEX-1/Thio-miniK was used as the template [13]. After isolation of appropriate fragments, *Thio-TR* and *KCNE3* were recombined using PCR. The target sequence was purified and digested by restriction endonucleases *Hin*dIII and *Nde*I with formation of the Thio-TR-KCNE3(*Nde*I/*Hin*dIII) fragment. To obtain expression vector pGEMEX-1/Thio-TR-KCNE3, three-component ligation of pGEMEX-1(*Nde*I/*Aat*II), pGEMEX-1(*Aat*II/*Hin*dIII), and Thio-TR-KCNE3(*Nde*I/*Hin*dIII) fragments was carried out.

*Escherichia coli* strain BL21(DE3)pLysS was transformed by expression vector pGEMEX-1/Thio-TR-KCNE3. The recombinant strain was grown at 37°C on M9 minimal salt medium in the presence of 100 μg/ml ampicillin on an Innova 44R shaker (New Brunswick, Germany). To obtain isotope-labeled KCNE3 derivatives, components of M9 medium $NH_4Cl$ and $C_6H_{12}O_6$ were replaced, respectively, with $^{15}NH_4Cl$ and $^{13}C_6H_{12}O_6$. To choose cultivation conditions, cells (50 ml) were grown in 500 ml Erlenmeyer flasks. After reaching optical density of 0.6 optical unit, the cell culture was divided into five 100 ml Erlenmeyer flasks and inducer isopropyl β-D-thiogalactoside (IPTG) was added once to final concentration 1.0, 0.25, 0.05, 0.01, and 0 mM, respectively, and cells were grown for 48 h at 13, 25, or 37°C with shaking at 250 rpm. Optimal temperature and IPTG concentration were determined by Tris-glycine electrophoresis. For preparative isolation, cell culture was grown in 2-liter Erlenmeyer flasks with 400 ml of culture in each. After achievement of optical density 0.6 optical unit, inducer IPTG was added to the cell culture to final concentration 0.01 mM and cultivation was continued for 48 h at 37°C and 250 rpm. Cells were collected by centrifugation for 30 min at 10,000*g*.

**Purification of KCNE3.** Cells from 1 liter of culture were resuspended in buffer A (50 mM Tris, pH 8.0, 0.2 M NaCl). The mixture was sonicated seven times, 30 sec each, with 5-min intervals on an ice bath and centrifuged for 30 min at 10,000*g*. The pellet of inclusion bodies was resuspended by sonication in buffer A containing 1% Triton X-100. The suspension was centrifuged under the same conditions. The supernatant was decanted, and the pellet of inclusion bodies was dissolved in buffer B (50 mM Tris, pH 8.0, 0.3 M NaCl, 1% lauryl sarcosine, 10 mM imidazole). For complete dissolution, the suspension was carefully mixed at room temperature for 3 h and centrifuged for 30 min at 10,000*g*.

Soluble protein was applied onto a column of Chelating Sepharose ($Ni^{2+}$) (15 ml) pre-equilibrated with buffer C (50 mM Tris, pH 8.0, 0.3 M NaCl, 0.1% lauryl sarcosine, 10 mM imidazole). The column was washed with buffer C containing 45 mM imidazole. The protein was eluted in buffer C containing 200 mM imidazole.

Protein-containing fractions were combined and diluted five times with buffer containing 50 mM Tris, pH 8.0, 0.3 M NaCl, and 0.1% lauryl sarcosine. The hybrid protein was cleaved with human thrombin at room temperature for 18 h. The enzyme was added to 0.3 unit per mg hybrid protein. Completeness of hydrolysis was estimated by Tricine gel electrophoresis [14].

After centrifugation for 1 h at 10,000*g*, the hydrolysis products were applied onto a column of Chelating Sepharose ($Ni^{2+}$) (15 ml) pre-equilibrated with buffer C. Unbound protein was applied on a column filled by hand with Diasorb C4 sorbent (12 ml) and washed with a linear acetonitrile gradient (10-70%) in acetate buffer (50 mM NaOAc, pH 6.0). After equilibration in buffer E (0.1% trifluoroacetic acid) containing 10% acetonitrile, the protein was eluted in a linear acetonitrile gradient (10-70% in buffer E). Protein-containing fractions were combined, immediately evaporated to half volume on an Eppendorf Concentrator 5301 apparatus (~30 min), frozen, and lyophilized.

**Preparation of KCNE3/detergent complexes and their analysis.** The lyophilized protein was dissolved in trifluoroethanol to homogeneous solution. In this case, protein concentration was approximately 0.1 mM. Dithiothreitol to final concentration 1 mM and aqueous solution with necessary detergent content were added to the protein solution. Final trifluoroethanol/water ratio was 1 : 1. The mixture was frozen in liquid nitrogen, and the solvents were removed by lyophilization. After lyophilization, samples were rehydrated in buffer containing 10 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM $NaN_3$.

Data on dynamic light scattering were obtained on a DynaPro Titan instrument (Wyatt Technology Corporation, USA) using 12 μl cells at 30°C. The data were acquired and processed out using the DYNAMICS 6.7.7.9 program (Wyatt Technology Corporation).

CD spectra were obtained on a JASCO-810 spectropolarimeter (Japan) equipped with a sample temperature control system. Spectra were recorded at 30°C using 0.01 cm quartz cell with a bandwidth of 2 nm, and the protein sample concentration was 1 mg/ml. The spectrum of buffer solution with detergent micelles was subtracted from the spectrum of the corresponding protein solution. The CD spectra were analyzed with the CDSSTTR program [15].

2D $^{1}H$-$^{15}N$ HSQC NMR spectra were obtained with an Avance 700 spectrometer (Bruker, Germany) with

operating frequency on protons of 700 MHz at 45°C. Spectra were processed in the TOP-SPIN program (Bruker).

## RESULTS AND DISCUSSION

The *KCNE3* gene was assembled of ten oligonucleotides partially overlapped along the sequence using PCR. The gene sequence was composed with account for translation codon usage in *E. coli* (Novagen Catalog 2002-2003). The *KCNE3* gene was expressed using plasmid pGEMEX-1/Thio via its attachment to the 3′-end of *E. coli* thioredoxin A gene using PCR [13]. Nucleotide sequences encoding six histidines (H6, histidine tag) and thrombin (TR) recognition (cleavage) site were introduced at the interface between the two genes. The histidine tag was provided for the possibility of protein purification by affinity chromatography, whereas the thrombin recognition site was specified for separation of the target protein from the carrier protein. Sequences Gly-Ser-Gly-Ser-Gly encoding flexible linkers (GS) were placed on both sides of H6. Their presence provided the availability of the histidine tag and the thrombin recognition site. The resulting plasmid DNA (pGEMEX-1/Thio-TR-KCNE3) directed synthesis of protein product Trx-GS-H6-GS-TR-KCNE3 (further Thio-TR-KCNE3) (Fig. 1).

**Bacterial culture.** *Escherichia coli* BL21(DE3)pLysS, widely used as a host strain, was chosen for obtaining cell-toxic proteins. To obtain maximal yield of soluble protein, bacterial growth temperature (37, 25, or 13°C) and optimal concentration of chemical inducer IPTG (0, 0.01, 0.05, 0.25, or 1 mM) were selected. The recombinant strain was grown on M9 minimal salt medium. Fusion protein Thio-TR-KCNE3 at all cell growth temperatures accumulated exclusively in inclusion bodies.

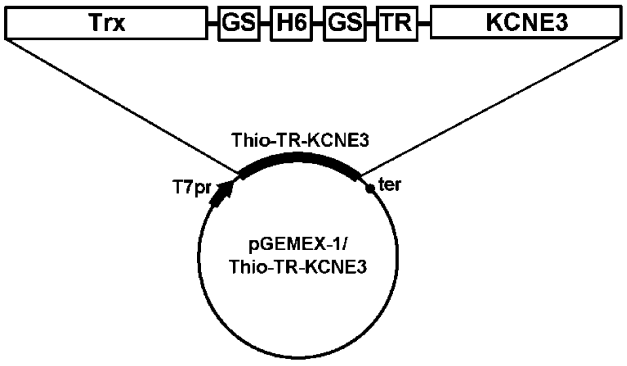


**Fig. 1.** Schematic representation of expression vector pGEMEX-1/Thio-TR-KCNE3 and of the corresponding protein product Thio-TR-KCNE3. Designations: T7pr, T7 promoter; ter, T7 terminator; Trx, thioredoxin A; TR, thrombin recognition site; H6, histidine tag; GS, flexible linker.

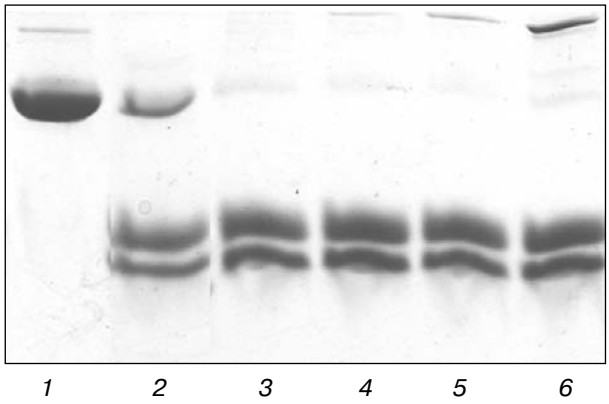


**Fig. 2.** Choosing conditions for Thio-TR-KCNE3 cleavage by thrombin. The protein was cleaved at different thrombin (activity units)/hybrid protein (mg) ratios: *1*) 0; *2*) 0.1; *3*) 0.3; *4*) 1; *5*) 3; *6*) 10.

Maximal yields of the fusion (approximately 100 mg from 1 liter M9 minimal medium) were observed in the case of recombinant strain growing at 37°C and 0.01 mM IPTG (data not shown).

**KCNE3 purification.** Protein accumulation in inclusion bodies increased the efficiency of its purification. The pellet of inclusion bodies was washed several times with a buffer containing 1% Triton X-100, which significantly increased their purity. Thio-TR-KCNE3 was solubilized in the ionic detergent lauryl sarcosine. This detergent was used during all following purification steps. Fusion protein was purified by immobilized metal affinity chromatography (IMAC) using Chelating Sepharose FF charged with nickel ions. The presence of the H6 sequence provided affinity to bivalent metal ions. Because of this, already at this stage a highly purified preparation of the hybrid protein was obtained.

Thrombin was used to separate KCNE3 from the partner protein [16]. This enzyme selectively hydrolyzes the peptide bond in the Leu-Val-Pro-Arg-Gly-Ser recognition site immediately after the arginine. To choose optimal hydrolysis conditions, the efficiency and specificity of Thio-TR-KCNE3 cleavage were checked at different enzyme/substrate ratios as well as at different protein, salt (NaCl), and imidazole concentrations. It was found that the ratio of 0.3 thrombin unit per mg Thio-TR-KCNE3 was optimal (Fig. 2). In this case, protein, salt, and imidazole concentrations in the reaction mixture did not influence the hydrolysis efficiency and specificity (data not shown).

The hydrolysis products were separated by IMAC. KCNE3 had no affinity to nickel ions, while trace amounts of uncleaved hybrid protein and of carrier protein, present in reaction mixture, were efficiently retained by the sorbent. KCNE3 was additionally purified by hydrophobic chromatography. Choosing this stage was due to the presence in the KCNE3 sequence of extended

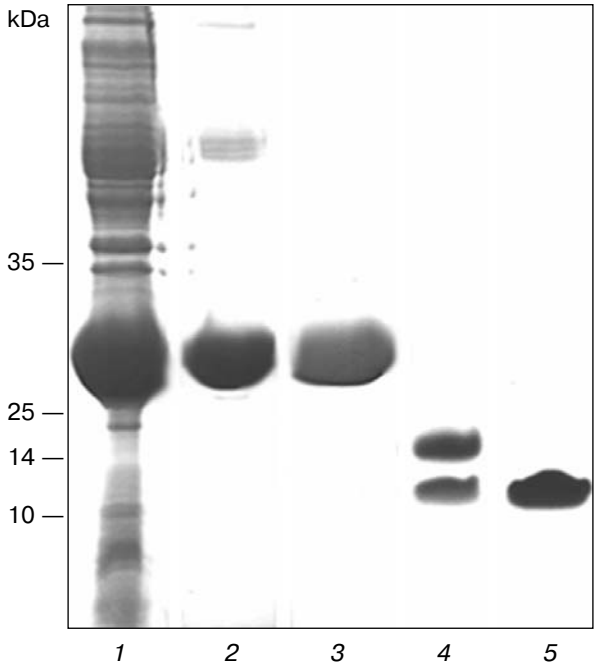


**Fig. 3.** Results of electrophoretic separation under denaturing conditions of samples at all stages of KCNE3 purification: *1*) cell lysate; *2*) inclusion bodies; *3*) purification by IMAC; *4*) Thio-TR-KCNE3 cleavage; *5*) purified KCNE3.

hydrophobic regions. The protein was chromatographed in two steps. First, the resin-bound KCNE3 was washed in a linear acetonitrile gradient in acetate buffer. Then the KCNE3 was eluted in a linear acetonitrile gradient in 0.1% trifluoroacetic acid (TFA). Rather rapid (during several hours) protein degradation took place at low pH values as detected by gel electrophoresis. Therefore, as soon as the chromatography was finished, acetonitrile and TFA were immediately removed from the sample by vacuum evaporation. Then the protein was lyophilized. The yield of KCNE3 as well as of its $^{15}N$- and $^{15}N$-/$^{13}C$-labeled derivatives was approximately 10 mg from 1 liter of M9 minimal salt medium. Figure 3 shows the results of electrophoretic separation under denaturing conditions of KCNE3 samples taken at different stages of purification.

**KCNE3 solubilization: screening the membrane mimetic environment.** Thorough selection of a medium imitating the protein native environment in the cell membrane is necessary in structural–functional investigations of membrane proteins. The most widespread medium for membrane protein investigations by high-resolution NMR spectroscopy is small micelles of detergents or short lipids [17, 18]. In this work optimal micelle composition for KCNE3 solubilization and obtaining NMR spectra suitable for interpretation was based on three main criteria: minimal size of supramolecular complexes consisting of micelles with incorporated protein; sample monodispersity and absence of protein oligomerization in the membrane-mimetic environment; high percent of α-helicity corresponding to the supposed native protein conformation within cell membrane.

For solubilization of KCNE3 and investigation of its behavior within micelles detergents with different length hydrophobic hydrocarbon chains and polar group structures dodecyl phosphocholine (DPC), lysomyristoyl phosphatidylcholine (LMPC), lauryl dimethylaminoxide (LDAO), lysomyristoyl phosphatidylglycerol (LMPG), and lysopalmitoyl phosphatidylglycerol (LPPG) were chosen. These detergents form micelles of different size and with different surface charge.

Dynamic light scattering was used for determination of micelle size and extent of sample dispersity. The secondary structure of the KCNE3 within micelles was determined by CD spectroscopy. With relation to the pos-

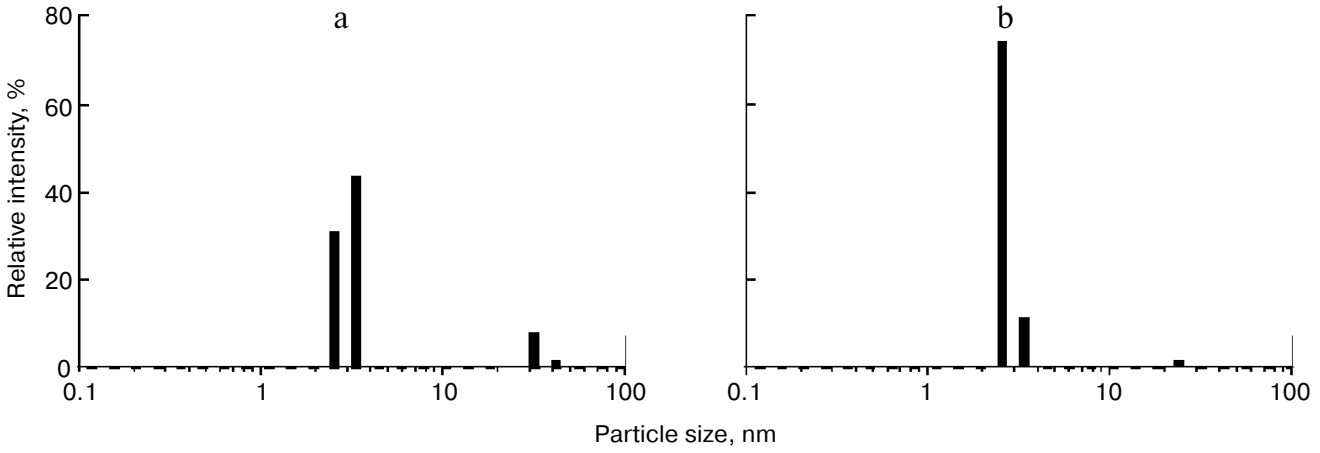


**Fig. 4.** Size distribution of protein/detergent complexes at various protein/detergent (mol/mol) ratios: a) 1 : 50; b) 1 : 120. Detergent is a mixture of DPC–LDAO (9 : 1 mol/mol). The relative intensity of scattered light is plotted on the ordinate axis.

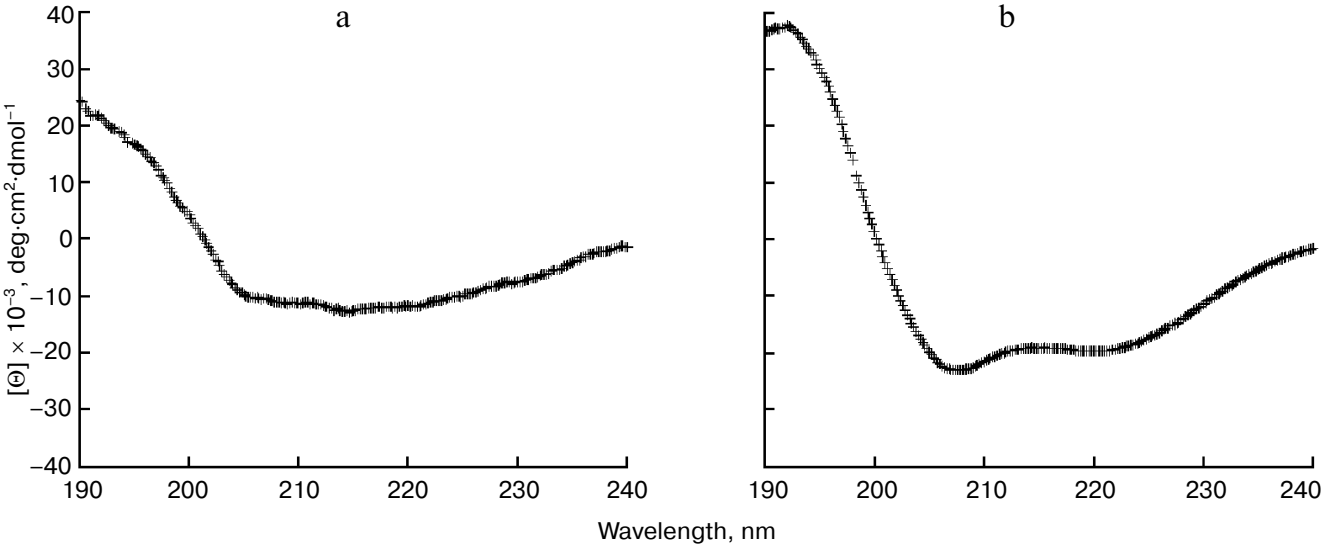


**Fig. 5.** CD spectrum in far UV region of KCNE3 in different detergent micelles: a) LDAO; b) DPC–LDAO (9 : 1 mol/mol) mixture. Protein/detergent ratio 1 : 120 (mol/mol). Ordinate axis, molar ellipticity.

sibility of structural investigations by NMR, the sample quality was estimated from the number of characteristic resolved signals, their widths, as well as by their doubling in two-dimensional $^{1}H$-$^{15}N$ NMR spectra.

It was shown by dynamic light scattering, that all of the selected detergents form relatively small micelles (radius below 3 nm) and can be used for solubilization of KCNE3 for subsequent structural investigations by NMR. The incorporation of KCNE3 into micelles of LMPC, LPPG, LMPG, LDAO, and DPC as well as into their mixtures was confirmed by the observed elongation of radii of the protein–detergent complexes relative to protein-free micelles.

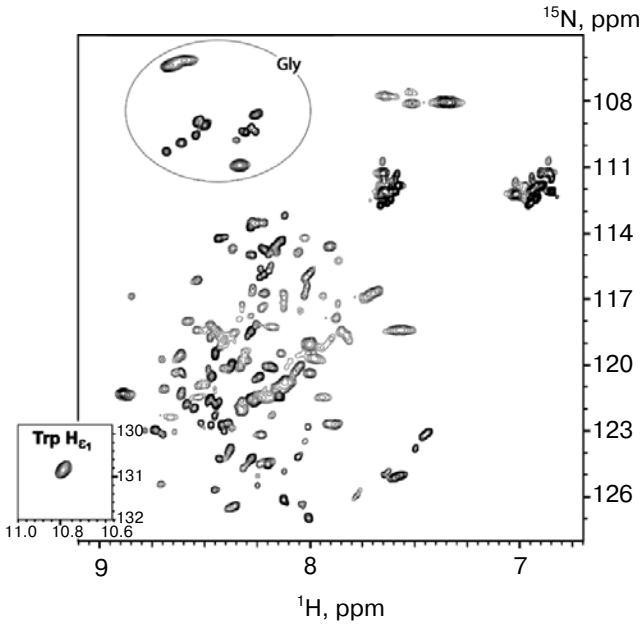


**Fig. 6.** $^{15}N$-HSQC NMR spectrum of KCNE3 in DPC/LDAO micelles. Protein/detergent ratio 1 : 120 (mol/mol), 45°C. The oval shows the region in which signals of all glycines are observed.

A problem in obtaining homogeneous micellar samples of membrane proteins is possible protein oligomerization during solubilization resulting in significant deterioration of NMR spectra. In this connection, the effect of the protein/detergent ratio on oligomerization during KCNE3 solubilization was studied. Data of dynamic light scattering in the case of KCNE3 solubilization by the DPC/LDAO mixture (9 : 1 mol/mol) at different protein/detergent ratios are shown in Fig. 4. As shown by the dynamic light scattering, in the case of protein/detergent molar ratio 1 : 50 mol/mol, corresponding to the protein/micelle ratio 1 : 1, large aggregates are present in the sample along with small micelles. When the detergent content was increased to achieve protein/detergent ratio 1 : 120 mol/mol, KCNE3 solubilization proceeds with formation of a monodisperse sample consisting of small micelles with incorporated KCNE3 monomer. This protein/detergent ratio was chosen for experiments for qualitative evaluation of NMR spectra.

Formation of the regular secondary structure elements in the KCNE3 polypeptide chain in DPC/LDAO (9 : 1 mol/mol) micelles was confirmed by CD spectroscopy. The shape of the CD spectra is typical for polypeptide chain in α-helical conformation (Fig. 5). According to the CD data, the α-helical content was 50-55%, which well agrees with the presumable presence of α-helical regions in the KCNE3 N- and C-terminal regions as well as in its transmembrane segment.

As shown by $^{1}H$-$^{15}N$ NMR, DPC/LDAO (9 : 1 mol/mol) micelles appeared to be optimal for solubilization of KCNE3 for further structural investigations. All seven signals from glycine residues and one from tryptophan were observed in the $^{1}H$-$^{15}N$ NMR spectra (Fig. 6). The total number of signals approximately coincided with that expected from the amino acid sequence of the protein. Signal widths were approximately 20 Hz for protons, whereas the KCNE3 signal width in LMPG micelles exceeded that for approximately 10 Hz. Dispersion of chemical shifts in $^{1}H$-$^{15}N$ NMR spectra suggests formation of elements of the regular secondary structures in KCNE3 in DPC/LDAO micelles and is indicative of the possibility of three-dimensional structure determination of KCNE3 by high-resolution heteronuclear NMR spectroscopy.

The proposed strategy can be used to obtain the target KCNE3 protein and its isotope labeled derivatives. The high quality and milligram amounts of protein preparations as well as small size of protein–detergent micelles will allow structural investigations of KCNE3 by NMR spectroscopy.

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