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Urea and guanidine salts as novel components for deep eutectic solvents

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ABSTRACT

We present guanidinium salts in combination with urea, used to prepare a new combination of deep eutectic solvents (DES). They have recently attracted much attention as "green" alternatives to conventional organic solvents in various fields including biophysical chemistry. These were subsequently used to test the stability and solubility of proteins in a water-free DES. We performed CD analyses in order to monitor global secondary and tertiary structural changes of cytochrome c in the presence of Guanidine HCl–urea. Formation of the melt form of DES at a room temperature was an effective way to store soluble protein in the denatured form for a long time. Consequent dialysis allows one to recover proteins into their native form.

1. Introduction

Room temperature ionic liquids (RTILs) are low melting-temperature salts (below 373 K) formed by bulky and asymmetric ions that hinder crystallization [1]. Properties of RTILs, such as viscosity, density, melting point or solubility in water can be tuned by changing either the anion or cation [2]. Deep eutectic solvents (DES) were reported by Abbott and co-workers [3] as extending model of ionic liquids. DES are generally defined as salts with a melting point less than 100 °C exhibiting unique characteristics: very low vapor pressures, non-flammability, high thermal and chemical stability, possibility of recycling, good solubility for a wide spectrum of compounds and high suitability for modifications [1,4–6]. DES have large depression of the freezing point of mixtures of salts with hydrogen donors (e.g. amines, amides, alcohols and carboxylic acids) [7]. The interaction of this hydrogen bond donor with the salt reduces the anion–cation electrostatic force, and thus, the freezing point of the mixture decreases. The enormously large number of salts and hydrogen bond donors that can be used to prepare DES compounds caused that there is no limit of combination of cations with anions. A classical example is the combination of choline chloride (m.p. 302 °C) with urea (m.p. 132 °C), forming a DES with a melting point of 12 °C [8].

Urea is a powerful protein denaturant as it disrupts the noncovalent bonds in the proteins. This group of chaotropic agents includes also ionic guanidine hydrochloride (GuHCl) and guanidine thiocyanate (GuSCN). These guanidine-based compounds are well known denaturing reagents and are used extensively in protein chemistry. Urea and guanidine salt denaturation curves are generally used to obtain knowledge of conformational stability of proteins [9–11]. The eutectic system GuHCl–GuSCN (eutectic point lies at 87 °C, 60 mol% of GuSCN) has been studied recently as well as several other combinations of guanidine salts such as GuFormate–GuSCN (eutecticum lies at 45 °C, 30 mol% of GuSCN) [12]. Eutectic points were not in a molar ratio as in the case of choline chloride and urea. The paper presents the results of some characteristic DES formed between urea and GuHCl as well as urea and GuSCN and its effect on the structure of ferricytochrome c detected by CD spectroscopy. These ionic liquids allow the high solubility of cytochrome c and bovine serum albumin in these non-aqueous medium and we discussed the possibility of using this type of DES for long-term storage of proteins at room temperature.

2. Materials and methods

Chemical denaturants, urea, GuHCl and GuSCN were raw materials of deep eutectic solvents and were obtained from Sigma-Aldrich without further purification. Stock solutions of GuHCl (5 M), GuSCN (5 M) and urea (9 M) were prepared by weighing commercial compounds and adding distilled water in the desired proportion to pH 7.0. A buffer solution, consisting of 10 mM sodium phosphate at pH 7.0 and pH 2.0, was used in some experiments and pH values of solutions were measured by pH meter HI 9017 by Hanna Instruments Company by Sensorex glass electrode. The eutectic mixtures were formed from the powders by heating and stirring two components GuHCl–urea at 70 °C and GuSCN–urea at 60 °C until homogenous liquid was formed. The molar ratios of eutectic compositions for CD measurements were found to be 1:2 (20 mM: 40 mM). The liquids were cooled at a rate of 1 °C/min and the freezing point was taken as the temperature at which the first solid

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began to form. The cyt c (99%) from horse heart and BSA (98%) was obtained from Sigma Aldrich. CD spectra were recorded with a Jasco J-815 spectropolarimeter equipped with a Julabo Peltier temperature control system (CDF-426S/15 model), using a cell of 0.2 mm path length over the wavelength range from 195–250 nm, 350–500 nm. Protein concentration was 5 mg/ml. CD signal-averaged over at least three scans, and baseline was corrected by subtracting the reference spectrum.

3. Results

We were able to prepare a new eutectic mixture with a lower eutectic point by replacing one of the guanidine salts in GuHCl–GuSCN for urea. Isobaric phase diagram (atmospheric pressure 101.325 kPa) of these binary systems (Fig. 1) shows a two-dimensional space, where the x-axis corresponds to the composition (in molar fractions) in accordance with the lever rule and the y-axis indicates the melting point temperature of DES (°C). Experimental determination of the phase diagram is usually carried out by thermal analysis, in which the temperature of the studied sample during its cooling at a constant speed of 2–5 °C/min is registered. When the system is cooled to the eutectic temperature it will preclude the solid phase. Temperature measurements of phase diagram were made on the basis of optical observations of crystals, using a Heidolph (Germany) heater with thermometer.

These values were confirmed under a light microscope (Optika Microscope) with heating and cooling of samples. Guanidine salts mixed with urea in a 1:2 molar ratio produce colorless liquid that freezes at 58 °C for GuHCl-urea and 47 °C for GuSCN-urea. For GuHCl-urea eutecticum is formed at a composition of 67 mol% urea which is the same as the eutectic recently reported for the choline chloride-urea system [8]. The lower melting point (42 °C) relates to GuSCN-urea mixture and occurs when the ratio of salt to hydrogen bond donor is 1:1.5 (60 mol% urea). The melting points of the mixtures are considerably lower than the melting points of either of the constituents (GuHCl melts at 185 °C, GuSCN at 120 °C and urea at 134 °C). Concentrations of the individual components at 1:2 molar ratio in the mixture GuHCl (5.7 M)–urea (11.4 M) and GuSCN (5 M)–urea (10 M) are sufficient for the complete denaturing of proteins (as in aqueous solutions).

In this report we demonstrate the ability of GuSCN–urea and GuHCl–urea (1:2) to dissolve higher amounts of two different proteins—bovine serum albumin (BSA) and cytochrome c (cyt c). BSA is a globular water soluble protein with molecular weight ~66 kDa and isoelectric point 4.7. BSA dissolves at least 260 mg per 1 ml in both DES. Higher concentrations could not be prepared in the form of real solution because of gel forming. The second studied biomacromolecule i.e. horse hearth cyt c is a heme protein with molecular weight ~12 kDa and isoelectric point 10.5. Cyt c was shown to dissolve at minimum 18 mg per 1 ml in both DES. Detection of higher concentrations of cytochrome c caused to observe solubility difficult because the solution was very colorful.

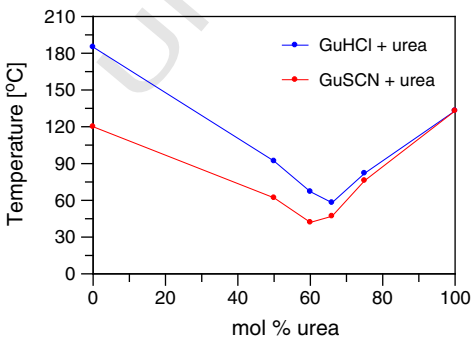

Fig. 1. Binary phase diagrams of guanidinium salts GuHCl (blue), GuSCN (red) and urea in different molar ratios. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

We performed CD analyses in order to monitor global secondary and tertiary structural changes of cyt c in the presence of GuHCl–urea. Although GuSCN has a stronger effect on protein denaturation as GuHCl (GuSCN–urea has a lower eutectic formation temperature), the disadvantage of its use in the far UV region is its stronger absorption than GuHCl. Therefore we used the GuHCl-urea system in the next measurements (the impact of DES on the CD spectrum of cytochrome c). However, usage of cuvettes with path length of 0.2 mm (two separate slides forming cuvette) was necessary for this DES. Cuvette constructed from two parts provides an advantage when measuring viscous samples. There is no problem loading the sample into the second enclosed cavity portion by spreading the sample evenly throughout the entire cuvette volume. Decreasing for CD signal of protein itself was offset by the high concentration of protein that dissolved as well in water as in DES. Cyt c allows deeper analysis of conformational changes by CD polarimetry as compared to BSA because heme is the internal probe characterizing the changes in the tertiary structure of the protein. Fig. 2 shows representative far- and near-UV CD spectra of cyt c in different conditions.

Fig. 2 (left) shows changes in the secondary structure of cytochrome c. Native cyt c in 10 mM phosphate buffer (pH 7.0 at 25 °C) shows classical dichroic minima at 208 nm and 222 nm, specific for the proteins with high amount of α-helicity. Water solutions of GuHCl (5 M) and urea (9 M) markedly replaced these minima by a large negative minimum below 210 nm suggesting an occurrence of a typical helix to random coil transition. The CD spectra of the cyt c in 10 mM phosphate buffer pH 2.0 at 25 °C indicate that the secondary structure is qualitatively similar with spectra of the cyt c in GuHCl–urea mixture at a temperature of 70 °C. The spectra suggest randomly coiled conformations in both cases, as well as in the individual components of a mixture. Difference in the spectra may be due to expanding the unfolding of cytochrome c. Fig. 2 (right) shows changes around the heme of cytochrome c. Negative peak at 416 nm and positive peak at 402 nm has been assigned for the tertiary structure corresponding to heme-polypeptide interaction close to the heme crevice and indicates native cyt c. In the presence of GuHCl–urea we observed the absence of negative Cotton effect at 416 nm and strong positive band with a maximum at 410 nm indicating a decoupling Met80 from binding to heme iron, like this observe for in all individual components of DES in water as well as for acidic pH 2.0 buffer.

The questions whether these denatured proteins in the melt form of DES can renaturate again is another problem arising here. Refolding of the solubilized proteins is initiated by the removal of the denaturant or reducing its concentration and allowing the protein to refold into the native conformation. The most often used approach here is the removal of the solubilizing agent (DES) by dialysis (as mentioned by other authors). Long-term storage of cytochrome c in GuHCl–urea DES (6 months) at room temperature, and after removal of DES in 10 mM phosphate buffer solution at pH 7 by means of dialysis, CD spectra (Fig. 2 red lines) demonstrated folding of cytochrome c into the native structure. During dialysis the concentration of the solubilizing agent decreases slowly what allows to refold the protein optimally. Dialyzed cyt c from GuHCl-urea solution was carried out in 10 mM phosphate buffer pH 7.0 at 25 °C and subsequently measured secondary (tertiary) structure and conformational stability. CD spectra of the refolded cyt c closely resemble those of the native cyt c, showing classical dichroic minima at 208 nm and 222 nm for secondary structure and negative peak at 416 nm and positive peak at 402 nm for tertiary structure (Fig. 2 — red line). The protein was in DES in melt form at RT for 6 month. Therefore, assuming the formation of solid melt in the vicinity of the protein stabilizes the denatured state and after removal of the denaturant lets his return to native form. These observations indicate that the protein could be stored at RT in the denatured state and consequently renaturate anytime becoming ready for further measurements.

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