Characterization of the synthetic compatible solute homoectoine as a potent PCR enhancer

Michael Schnoor\textsuperscript{a,b}, Peter Voß\textsuperscript{b}, Paul Cullen\textsuperscript{a,c}, Thomas Böking\textsuperscript{a}, Hans-Joachim Galla\textsuperscript{b}, Erwin A. Galinski\textsuperscript{d}, Stefan Lorkowski\textsuperscript{a,b,*}

\textsuperscript{a} Institute of Arteriosclerosis Research, University of Münster, Germany
\textsuperscript{b} Institute of Biochemistry, University of Münster, Germany
\textsuperscript{c} Ogham GmbH, Münster, Germany
\textsuperscript{d} Institute of Microbiology and Biotechnology, University of Bonn, Germany

Received 28 July 2004

Abstract

Different substances such as dimethyl sulfoxide, tetramethylene sulfoxide, 2-pyrollidone, and the naturally occurring compatible solute betaine enhance PCR amplification of GC-rich DNA templates with high melting temperatures. In particular, cyclic compatible solutes outperform traditional PCR enhancers. We therefore investigated the effects that cyclic naturally occurring ectoine-type compatible solutes and their synthetic derivatives have on melting temperature of double-stranded DNA (dsDNA) and on PCR amplification of different templates. LL-Ectoine, betaine, and derivatives of LL-ectoine decreased, whereas \textbeta-hydroxyectoine increased, the melting temperature of dsDNA. The ability to decrease the melting temperature was greatest for homoectoine, a new synthetic derivative of L-ectoine. Furthermore, compatible solutes, especially homoectoine, enhanced PCR amplification of GC-rich DNA (72.6\% GC content; effective range: 0.1–0.5 M).

\textcopyright 2004 Elsevier Inc. All rights reserved.

Keywords: Compatible solutes; PCR enhancer; Homoectoine; Betaine; DNA melting

The polymerase chain reaction (PCR) developed by Mullis and colleagues [1] is one of the most important methods in all of the biological sciences. The initial step in each PCR cycle is the separation (melting) of double-stranded DNA (dsDNA) into single strands by heat denaturation. This is often a critical step, in particular when the melting temperature of the template is high (>90 °C). Except for the length, the melting temperature depends on the base pair composition of the template [2] and on electrolytic effects resulting from the salt concentration and the ion types in the reaction buffer [3]. Limitations in PCR caused by high melting temperatures can be counterbalanced by the addition of different compounds such as dimethyl sulfoxide, tetramethylene sulfoxide (sulfolane), 2-pyrollidone, and naturally occurring compatible solutes such as betaine [4–7]. Compatible solutes are small organic zwitterionic compounds that do not negatively affect cellular processes [8]. Halophilic prokaryotes accumulate these protecting molecules in their cytoplasm to regulate an osmotic gradient, enabling them to survive in extremely saline habitats.

Betaine, L-ectoine, and \textbeta-hydroxyectoine are well-known examples of this class of molecules [8]. It is not known in detail how compatible solutes mediate protection of cells or how these compounds interact with macromolecules although several hypotheses have been suggested [9–12]. Ten years ago, Rees et al. [13] demonstrated that betaine eliminates the dependence of dsDNA melting on the base pair composition. A few years later, it was shown that the addition of betaine...
enhances amplification of GC-rich templates [14]. An additional effect of compatible solutes that improves the PCR is their ability to protect proteins against stressors such as heat and repeated freeze/thaw cycles [15–17]. This ability probably stabilizes the polymerase during denaturation steps of the PCR.

The aim of this study was to investigate the influences of naturally occurring cyclic ectoine-type compatible solutes and a synthetic derivative called homoectoine on dsDNA melting and on the PCR amplification of GC-rich templates.

Materials and methods

Compatible solutes. Betaine was purchased from Sigma, l-ectoine and β-hydroxyectoine were provided by Bitop. Synthetic derivatives of l-ectoine were synthesized from ortho-acetic acid-trimethylester and varying diamino carboxylic acids according to Koichi et al. [18] (Fig. 1; Table 1). Decarboxyectoine was synthesized from acetamidine hydrochloride and diamino propane: 50 g acetamidine hydrochloride was dissolved in 250 ml methanol. After adding 45.4 ml diamino propane, the solution was boiled at 70 °C. The ammonia generated was neutralized and disposed of. The methanol was removed and the product was recrystallized from methanol. In this study, we focused on homoectoine because it can be produced in sufficient yield as a pure enantiomer at low cost.

![Chemical structures of compatible solutes. (1) Betaine; (2) l-ectoine (1,4,5,6-tetrahydro-2-methyl-pyrimidine-4-carboxylic acid); (3) β-hydroxyectoine (3,5,6-trihydroxy-1,4,5,6-tetrahydro-2-methyl-pyrimidine-4-carboxylic acid); (4) decarboxyectoine (4,5,6,7-tetrahydro-2-methyl-pyrimidine); and (5) homoectoine (4,5,6,7-tetrahydro-2-methyl-1H-[1,3]-diazepine-4-carboxylic acid). Decarboxyectoine and homoectoine are new synthetic derivatives of l-ectoine.](Image 1)

Cell culture. Human coronary smooth muscle cells were purchased from BioWhittaker. Cells were cultured using the SmGM2 BulletKit (BioWhittaker) in medium containing 5% fetal bovine serum (Cytogen). When confluent, the cells were grown 24 h in MCDB media (Sigma) supplemented with penicillin/streptomycin and l-glutamine (ICN Biomedicals), sodium hydrogen carbonate (Sigma), and 1% fetal bovine serum.

Template preparation. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). After DNase I treatment, cDNA was synthesized using oligo(dT) primers and SuperScript II (Invitrogen) as described [19,20]. To obtain dsDNA templates, PCRs were performed using 1.25 U HotStar Taq polymerase (Qiagen), 0.25 mM dNTPs, and 200 nM of each primer in a PTC-200 PCR cycler (MJ-Research). The following primers (Invitrogen) were used to generate a 477 bp GC-rich template (α-chain mRNA of type XVIII collagen, COL18A1) and a 139 bp (for melting temperature measurements) or 82 bp (for PCR amplification efficiency experiments) GC-poor template (14 kDa signal recognition particle, SRP14): COL18A1 forward: 5'-TCTACGTC CGCGTCAGAA-3'; COL18A1 reverse: 5'-GGCGCACGATGCTG TACAG-3'; SRP14(139) forward: 5'-GGTTCGTGGTTCCCCCACA GT-3'; SRP14(139) reverse: 5'-ACCATGACGACAGACAAATT- 3'; and SRP14(82) forward: 5'-AGCAGTGTGAGCTCAGGAC- 3'; SRP14(82) reverse: 5'-TCAAGCCTACCTGTTGCTCTA-3'.

PCR conditions were as follows: The initializing step was the activation of the HotStar Taq polymerase at 95 °C for 15 min followed by 40 cycles with 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 1 min followed by a final elongation at 72 °C for 10 min. To enable PCR amplification of the highly GC-rich COL18A1 cDNA, 1 M betaine was added. Following amplification, products were separated by agarose gel electrophoresis. Bands of interest were gel extracted (QIAquick Gel Extraction Kit, Qiagen) and their concentration was measured spectrophotometrically (BioPhotometer, Eppendorf).

Melting temperature determination. For determination of melting temperatures in the presence of different concentrations of salt ions and compatible solutes, the following mixtures were used: (i) low salt mixture: 10 ng dsDNA template and 0.25× HotStar Taq polymerase PCR buffer (Qiagen) supplemented with 1 μl of a 1:2000 SYBR Green dilution (Stratagene) in TE buffer; (ii) high salt mixture: 10 ng dsDNA template and 1× HotStar Taq DNA polymerase PCR buffer (Qiagen) supplemented with 1 μl of a SYBR Green 1:2000 dilution (Stratagene) in TE buffer. The compatible solutes l-ectoine, β-hydroxyectoine, betaine, and homoectoine were added in final concentrations of 0.1–4.5 M. Melting points were measured using an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Buffer solutions without compatible solutes served as controls. This procedure was established and shown to be reliable using different amounts of the dsDNA templates.

PCR amplification of templates. To determine the influence of compatible solutes on PCR amplification, PCRs were performed using cDNA, PCR conditions, and primers as described above. Compatible solutes were added in the following final concentrations: 0.00, 0.10, 0.25, 0.50, 0.75, 1.00, and 1.50 M in the case of l-ectoine, β-hydroxyectoine, and homoectoine, and 0.00, 0.50, 0.75, 1.00, 2.00, 3.00, 4.00, and 4.50 M in the case of betaine. PCR products were analyzed by agarose gel electrophoresis.

Spectrophotometrical analysis. The following complementary GC-rich (70% GC) and AT-rich (70% AT) oligonucleotides (Invitrogen)
were used. GC-rich forward: 5'-ACGTGGACCGTGACGTTCGGCAGACGTTCCAACTGACCTCCCTAGAAATCATGGGTCGAATTCATGAACATTGATACATG-3'; GC-rich reverse: 5'-CGACGGTAGCCGGACTGGACG-3'; AT-rich forward: 5'-CATGTATCAATGTTCATGAATCATTGCTAATTCAGTCCGGCTACCGTCGGACGTCCACGGTCGCACGT-3'; AT-rich reverse: 5'-ATGAACTGAATGCAATGATTCATGAACATTGATACATG-3'. Oligonucleotides with reverse complementary sequences were mixed in equal amounts, denatured at 95 °C, and cooled to allow annealing. Formation of dsDNA was verified by photometrical analyses of the occurring hypochromic effect [21]. DNA solutions were diluted to a final concentration of 1 μM using water or 3 M betaine solution. After denaturation at 95 °C, samples were cooled. Betaine solutions or water containing non-denatured dsDNA and betaine solutions or water without any DNA served as controls. Wavelength scans were performed in a Uvikon 922 spectrophotometer (Kontron Instruments).

Results

Effect of compatible solutes on dsDNA melting temperature

We studied the effect of betaine, L-ectoine, β-hydroxy-ectoine, and the newly synthesized homoectoine on the melting temperature of GC-rich (COL18A1) and GC-poor (SRP14) templates. To investigate whether salt ions influence this effect, we performed measurements in two PCR buffer concentrations (Fig. 2, Table 2). β-Hydroxy-ectoine slightly increased the melting temperature of SRP14 DNA at both buffer concentrations (Figs. 2A and B). In the case of COL18A1 DNA, an increase was observed in the 0.25× PCR buffer, whereas a slight decrease in the melting temperature was observed in the 1× PCR buffer (Figs. 2C and D). Betaine, L-ectoine, and homoectoine all decreased the melting temperature of both DNAs at both buffer concentrations. The decrease of melting temperatures increased with the concentration of the compatible solutes added to the PCR buffer (Fig. 2). It is obvious that in both buffers and at all concentrations homoectoine had the greatest effect on dsDNA melting temperature. In contrast to betaine, homoectoine showed great effect even at very low solute concentrations. At concentrations of 1 M of solute, the

![Fig. 2. Effects of solutes on the melting temperatures of dsDNA. (A) Ten nanograms of SRP14 dsDNA in 0.25× PCR buffer. (B) Ten nanograms of SRP14 dsDNA in 1× PCR buffer. (C) Ten nanograms of COL18A1 dsDNA in 0.25× PCR buffer. (D) Ten nanograms of COL18A1 dsDNA in 1× PCR buffer. See text for details. Closed boxes, betaine; open boxes, β-hydroxyectoine; open circles, L-ectoine; and closed circles, homoectoine.](image-url)
decrease in COL18A1 dsDNA melting temperature with homoectoine was about four times greater in 0.25× buffer and about five times greater in 1× buffer compared to the effect of betaine. This effect was even greater with SRP14 dsDNA. The decrease in SRP14 dsDNA melting temperature caused by addition of homoectoine was 13.5 times greater in 0.25× buffer and 10 times greater in 1× buffer in comparison to the effect of betaine. Apparently, the GC-discriminating effect of homoectoine is not as pronounced as with betaine. In absolute terms, the decreasing effect that betaine and especially homoectoine exert on the melting point of dsDNA was greater in the lower concentrated buffer. In case of l-ectoine, the buffer concentration did not influence the decrease of the melting temperature of dsDNAs.

Effects of compatible solutes on PCR amplification

To examine how the compatible solutes affect DNA amplification by PCR, we performed RT-PCRs of a GC-rich (72.6%; COL18A1) and a GC-poor (39.6%; SRP14) cDNA template using PCR buffers supplemented with compatible solutes in different concentrations. The amplification of the GC-poor SRP14 template was achieved without addition of any compatible solute, whereas no amplification of the GC-rich template was achieved without addition of solutes. The effects that the solutes had on the amplification of the GC-rich templates correlated with the observed effects of the compatible solutes on the melting temperature of the COL18A1 DNA template. As expected from the finding that β-hydroxyectoine did not lower the melting temperature of dsDNA, addition of this compound did not result in PCR amplification of the GC-rich template (Fig. 3). As was expected from the ability of l-ectoine and betaine to lower the melting temperature of dsDNA, the addition of these compounds to the PCR facilitated amplification of GC-rich templates. However, PCR supplemented with l-ectoine and betaine resulted in amplification of the band of interest and in unspecific amplification of a contaminating fragment of about 250 bp at some solute concentrations. By contrast, PCRs supplemented with homoectoine resulted only in amplification of the band of interest at much lower concentrations of this compound compared to l-ectoine and betaine.

Limitations in solute-supported PCR amplification

With every compatible solute we observed a cut off in PCR efficiency that occurred when the solute concentration was higher than 2 M for betaine or 0.5 M for l-ectoine and homoectoine. At this or higher solute concentrations no PCR product was obtained (Fig. 3). The effective range for betaine was between 0.5 and 2 M (highest specificity) whereas much lower concentrations were needed in the case of l-ectoine (0.25–

![Fig. 3. Effects of solutes on PCR amplification of GC-rich cDNA. Amplification of COL18A1 cDNA (72.6% GC content) was only possible after addition of solutes. Betaine, l-ectoine, and homoectoine enhanced amplification at certain concentrations, whereas β-hydroxyectoine had no effect. NTC, no template control; STD, 1 kb DNA ladder.](image)

![Fig. 4. PCR limitations caused by addition of solutes can be counterbalanced by increasing primer, template or polymerase concentrations. Without betaine and with addition of up to 1.8 M betaine the SRP14 template could be amplified using the standard protocol. At betaine concentrations higher than 1.8 M, amplification could be achieved only if (i) 5-fold template concentration, (ii) 10-fold primer concentration, or (iii) 5-fold Taq polymerase concentration was used. By contrast, a 10-fold increase in dNTP concentration had no effect. NTC, no template control; STD, 1 kb DNA ladder.](image)
SRP14 template was inhibited by betaine concentrations of 1.8 M or higher. This indicates that in addition to the lowering effect of compatible solutes on dsDNA melting temperatures, competition might occur between primers, template, DNA polymerase, and solutes during the annealing of the primers to the template DNA or during formation of the synthesis complex consisting of primer, template, and DNA polymerase. To control this, we performed PCRs with 10-fold primer concentrations, 5-fold template concentration or 5-fold polymerase concentration (Fig. 4), conditions that should tip the reaction towards annealing and amplification. Using these conditions, PCR products were detected at betaine concentrations up to 2.2 M. This was a more than 20% increase in betaine tolerance compared to the standard conditions. As control, we performed a PCR with 10-fold dNTP concentration. This should not influence the annealing process but is known to enhance PCR efficiency. We found that a 10-fold dNTP concentration did not improve the PCR amplification (Fig. 4). To investigate whether betaine interacts with ssDNA and prevents renaturation of dsDNA, we performed spectrophotometrical analyses. Due to the hypochromic effect of dsDNA, denatured single-stranded DNA (ssDNA) shows a significantly higher absorbance than dsDNA. Thus, if a stabilization of the single DNA strands occurs in the presence of betaine after denaturation of the dsDNA, samples should show a higher absorption as controls without betaine. However, our measurements revealed no inhibition of renaturation to dsDNA (data not shown).

Discussion

The effect of compatible solutes on PCR amplification correlate well with the influence of these solutes on the melting temperature of dsDNA, supporting the speculation that this is the most important effect of the solutes with respect to enhancement of PCR amplification. The effect of betaine, l-ectoine, and homoectoine on the melting temperature was greater for GC-rich templates than for GC-poor templates. These results are consistent with the findings of Rees et al. [13] and Henke et al. [14], who showed that betaine eliminates the base pair composition dependence of dsDNA melting with respect to the GC content and enhances the amplification of GC-rich templates. In our hands, the synthetic compound homoectoine lowered the melting temperature of GC-rich dsDNA to the greatest extent and enhanced the specificity of PCRs to 100% over the entire effective range. With betaine and l-ectoine, a contaminating product of about 250 bp appeared at some concentrations. These findings agree with results presented by Lapidot et al. [22], who found contaminating products in PCRs supplemented with l-ectoine.

Because the loss of amplification at high solute concentrations could, in the case of betaine, be overridden by increased primer, template or polymerase concentrations but not by increased dNTP concentrations, we speculate that betaine affects primer annealing and/or formation of the synthesis complex consisting of ssDNA, oligonucleotide, and polymerase. Several lines of evidence support this hypothesis. In one study, increasing primer length counteracted a decrease in priming specificity seen at high concentrations of the compatible solute proline, indicating that compatible solutes reduce the affinity of oligonucleotides to DNA [23]. In addition, Malin et al. [24] showed that l-ectoine and β-hydroxyectoine induce a change in the structure of DNA such that many restriction endonucleases were no longer able to cleave it. Thus, addition of compatible solutes to a PCR may change DNA conformation in a way that influences primer annealing and binding of the polymerase. In addition, zwitterionic compounds such as glycine increase the dielectricity of a solution, resulting in a decrease of ionic interaction [25]. According to Manning's [26] counterion condensation theory, this explains the counteracting effects of most solutes on the increase in DNA melting temperature caused by cations such as Mg²⁺ and Na⁺ [3]. It is not clear how and to what extent these effects enhance specificity and efficiency of PCR amplification, but one possibility is that the interaction of the solutes is not limited to either DNA or protein but also affects the amplification machinery per se.

A decrease in dsDNA melting temperature is not a general effect of compatible solutes since this effect was not seen for β-hydroxyectoine. The results suggest that the chemical group in β-position to the carboxylic group plays an important role in the interaction of the solutes with DNA. Decarboxylation of l-ectoine, i.e., a change from the zwitterionic molecule to a cationic molecule, significantly attenuated this effect (data not shown), indicating that the carboxylic group or the zwitterionic structure is important for lowering the melting temperature. Since the more hydrophobic homoectoine had the greatest effect on dsDNA melting even at low solute concentrations, it is possible that the hydrophobicity plays a role in the interaction of compatible solutes with DNA. To understand the exact mode of action of compatible solutes, systematic studies of betaine and ectoine derivatives are required. In particular, the impact of different kinds of substituents on β-position to the carboxylic group and the effect of cationic and anionic solutes are of interest.

It is possible that the interaction of ssDNA and compatible solutes slows primer annealing and/or stabilizes ssDNA. Both abilities would tend to increase the specificity of the amplification. However, our spectrophoto-
metrical wavelength scans in the presence of high betaine concentrations revealed no inhibition of DNA reannealing. We therefore conclude that betaine, at least, does not stabilize ssDNA.

In conclusion, we achieved by far the best results with our new compound homoecotine which lowered melting temperatures of dsDNA most efficiently and was particularly effective in PCR amplification of the GC-rich template. In our hands, homoecotine worked at much lower concentrations than betaine and without unspecific amplification, so that it may be useful in tricky clinical and forensic PCR applications.

Acknowledgment

M.S., T.B., S.L., and P.C. were supported by the European Union (Grant No. QLG2-CT-1999-01007) [27].

References