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Thermostabilization and thermoactivation of thermolabile enzymes by trehalose and its application for the synthesis of full length cDNA

(chaperonin-like molecules/polyols/enzyme thermostability/reverse transcriptase)

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ABSTRACT The advent of thermostable enzymes has led to great advances in molecular biology, such as the development of PCR and ligase chain reaction. However, isolation of naturally thermostable enzymes has been restricted to those existing in thermophylic bacteria. Here, we show that the disaccharide trehalose enables enzymes to maintain their normal activity (thermostabilization) or even to increase activity at high temperatures (thermoactivation) at which they are normally inactive. We also demonstrate how enzyme thermoactivation can improve the reverse transcriptase reaction. In fact, thermoactivated reverse transcriptase, which displays full activity even at 60°C, was powerful enough to synthesize full length cDNA without the early termination usually induced by stable secondary structures of mRNA.

The usefulness of thermostable enzymes is indisputable; they allowed the development of outstanding techniques such as PCR and ligase chain reaction (1, 2). However, the isolation of thermostable enzymes has been restricted to those existing in thermophylic organisms. To expand the availability of thermostable enzymes, we explored a completely new way—the thermal stabilization of those enzymes that are normally thermolabile by the addition of structure stabilizing molecules. In particular, we explored the properties of molecules that are normally involved in heat shock response, and we anticipated being able to confer thermal stability to enzymes. Among them, there is the disaccharide trehalose. Trehalose synthesis is induced by heat shock in the *Saccharomyces cerevisiae* yeast (3), suggesting its possible role in this response and in desiccation tolerance (4). In fact, yeast mutants defective in trehalose synthesis show a significant reduction in thermotolerance (5). It has been reported that enzymes could be protected from irreversible heat aggregation–heat denaturation *in vitro* by trehalose, suggesting its chaperonin-like function (6). Trehalose also has been used to confer stability to dried enzymes (7).

In the present study, we discovered that trehalose can be used as a reaction additive to stabilize or stimulate enzymatic activity at unusually high temperatures, enabling the use of thermosensitive enzymes as though they would be thermostable. This property should be useful for converting a wide range of thermosensitive enzymes to thermostable and thermoactive ones for wide applications in biological, medical, and industrial fields.

To show the power of trehalose-mediated thermal activation, we subsequently applied this new method to the synthesis of full length cDNA. The major obstacle to preparing high quality cDNA libraries has been the low efficiency of reverse transcriptase (RT) to synthesize full length cDNA, which is due to the strong secondary structure of mRNA, which cause the RT to stop the synthesis and subsequently to be released from the hybrid mRNA/incomplete cDNA. To overcome problems associated with the secondary structure of mRNA, both denaturing of sample before the reaction and increased temperature reaction would be advisable. However, attempts to overcome this problem by heat destabilization of the secondary structures of mRNA before reaction or treatment of mRNA with methylmercury hydroxide (8) were not successful, especially to obtain full length cDNA from very long transcripts (9). Although the increase of the reaction temperature might be useful for destabilizing the secondary structures of mRNA for high efficiency, full length cDNA synthesis (9), no thermostable enzymes with RT activity have been reported except for *Tth* DNA polymerase (10). However, this enzyme shows RT activity only in the presence of Mn^{2+}, which readily degrades RNA before full length cDNA can be synthesized. In this report, we demonstrate the usefulness of trehalose to render a Moloney murine leukemia virus RT thermostable, which becomes then very highly effective to synthesize full length cDNAs.

MATERIALS AND METHODS

Assay of Thermal Activity of Enzymes. Enzymatic reactions were performed in the recommended buffer, with and without the addition of trehalose (or other sugars where specified) at 0.6 M unless otherwise specified. Samples were incubated at the recommended temperature (37°C, except 40°C for agarase) and as specified in the text. The activities of restriction nucleases in the presence or absence of trehalose were tested on λ DNA by using 0.5–1 units to follow the activation and 1–3 units/μg DNA to follow the stabilization. DNase (RQ1 RNase-free DNase, Promega) was tested by using 0.025 units/μg DNA. RNase I (Promega) was tested by digesting 10 μg of RNA with 0.65 units of RNase I for 1 h. Changes in the activity of nucleic acid-cleaving or –degrading enzymes were followed by agarose electrophoresis and densitometric analysis of the intensity of the produced bands. The activity of agarase (New England Biolabs) was tested by measuring the residual amount of undigested agarose after incubating 200 μl of 0.8% low melting point agarose with 0.8 units of agarase for 1 h. Polynucleotide kinase was tested by measuring the incorporation labeling by γ[^{32}P]ATP on a 30-mer. Klenow fragment activity was tested by measuring the efficiency of preparation a probe by random primer technique (11). RNA polymerase activity was measured by preparing *in vitro* transcripts.

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Abbreviation: RT, reverse transcriptase.
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RT Reaction. Template RNAs to test RT activity were obtained by *in vitro* transcription. The 5-kb *in vitro* transcript was obtained by from pBluescript II SK (Stratagene), and the reverse transcription reaction was primed with SK primer (5′-CGCTCTAGAACTAGTGGATC-3′). The 5.5- and 10-kb *in vitro* transcripts were obtained from pZL1 (GIBCO/BRL) and subsequently primed with 5′-GAGCAGCTGGGCCCT-TAA-3′.

The RT reaction used 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM ditiotreitol, 0.05 μg/μl BSA, 3 mM dNTPs (sodium salt), 0.2 μl of α [^{32}P]dGTP, 1 μg of template RNA, 400 ng of specific primer, and 200 units of Superscript II in a volume of 20 μl. The RNA and primer mixture initially were heated to 65°C before addition of the other reagents. The reaction mixtures containing 0.6 M trehalose also had 15% (vol/vol) glycerol. Reactions and temperatures were as follows: for the reaction in Figs. 2*A* and 3, after 2′ of annealing at 37°C, samples were transferred directly to the final temperature of the reaction. For the reactions in Fig. 2*B*, primers were annealed for 5′ at 37°C and followed by reaction in the thermal cycler at the final temperature specified for each sample. The temperature–cycle reactions were performed in a MJ Research (Cambridge, MA) thermal cycler as follows: After annealing of the sample at 37°C for 5 min and extension at 45°C for 5 min, the samples were incubated at 60°C for 2 min followed by 55°C for 2 min, for 10 cycles. cDNA synthesis was followed by alkaline gel electrophoresis (11).

RESULTS

Three Different Effects of Trehalose. We analyzed the actions of trehalose on enzymes *in vitro* and categorized them into three types. The first is the thermoprotection effect, which we define here as recovery of the original enzymatic activity after a short exposure to high temperature. This property was proved previously by testing the enzymatic activity when returning the temperature to a normal value (usually 37°C) after a heat shock (6). In this paper, we newly discovered the following two effects— thermostabilization and thermoactivation—by testing the enzymatic activity in the course of the heat shock. We thus define here the thermostabilization effect, which corresponds to the presence of enzymatic activity at unusually high temperatures, at which enzymes are normally inactive. Additionally, we define the thermoactivation effect that corresponds to an unexpectedly higher activity of some thermostabilized enzymes, if compared with their activity under standard conditions. As in the first step, we used the restriction enzyme *Sty*I as the example of thermostabilization, and DNaseI and the restriction enzyme *Nco*I were used as examples of thermoactivation. The results are shown in Fig. 1: *Sty*I restriction enzyme showed greatly reduced activity at 50°C or higher temperatures under standard buffer condition. However, in the presence of 0.6 M trehalose, *Sty*I showed almost normal activity at 50°C and residual activity even at 55°C (Fig. 1*A*). Trehalose was used at 0.6 M because this value is close to the highest concentration measured in yeast cells (5). Higher concentrations of trehalose did not further stabilize enzymes in preliminary experiments. Fig. 1*B* shows the results of thermoactivation, as in the case of DNaseI and *Nco*I. In the presence of trehalose, the optimum temperature of DNaseI became 50°C, with high observed activity still at 55°C and 60°C, at which the enzyme almost was inactivated completely in the absence of trehalose. Thermoactivation of the restriction enzyme *Nco*I also was noted. The highest activity was observed between 45 and 55°C in its absence but between 50 and 60°C after the addition of trehalose, with activity still being observed at 62.5°C (Fig. 1*B*). In the case of both DNaseI and *Nco*I, trehalose increased the optimal temperature of 5–10°C and enhanced their activity by 2- to more than 10-fold (Fig. 1*B*).

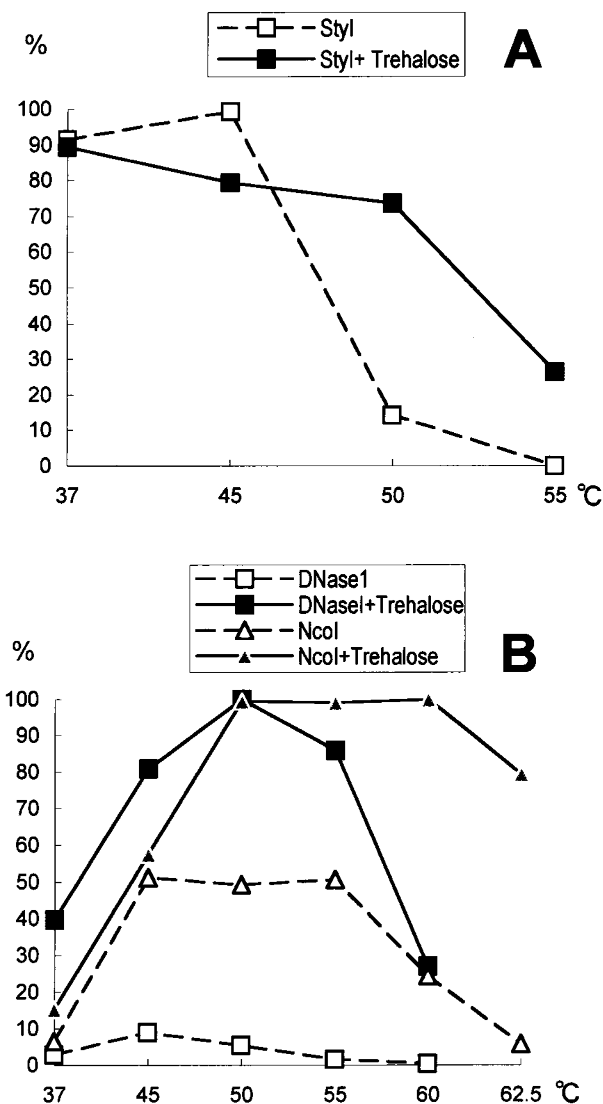

FIG. 1. Differences between stabilization and activation. (*A*) Stabilization: relative activity of *Sfy*I at various temperatures in the absence or presence of trehalose. (*B*) Activation: relative activity of DNaseI and *Nco*I restriction enzyme at the indicated temperatures in the absence or presence of trehalose. °C axis, temperature of incubation; % axis, relative activity (arbitrary units). Data are representative of three independent sets of experiments.

These effects of trehalose on several enzymes commonly used in molecular biology are summarized in Table 1. Several enzymes, such as MMLV RT, *Sty*I, *Eco*RI, *Nco*I, DNaseI, RNaseI, *Nde*I, *Pvu*II, and *Pst*I, were thermostabilized, and a subset of them also could be thermoactivated. The temperature of thermostabilization varied case by case, extending up to 10° from the starting temperature. Some enzymes, such as β-agarase, polynulceotide kinase, T3 RNA polymerase, and Klenow fragment DNA polymerase, for which further analysis was not done, were inhibited partially by trehalose at the 0.6-M concentration (Table 1). Some enzymes, such as *Dra*I, *Hinc*II, and *Hin*dIII, were not affected.

Thermostabilization and Thermoactivation of RT. We tried using trehalose to thermostabilize and thermoactivate RT with *in vitro* transcripts 5, 5.5, and 10 kb in size as substrates. The reaction containing trehalose also contained glycerol at a 15% concentration. Although glycerol does not confer any detectable thermal stability to RT, we found it slightly stimulatory when added to the reaction containing trehalose during preliminary tests (not shown). Compared with the control reac-

Table 1. Summary of trehalose effect on several enzymes

Enzyme	M	Stabilization	Activation	>90% activity	>30% activity	Notes
MMLV RT	0.6	++	+	**60**/*50*	**60**/*50*	a
*STy*I	0.6	++	0	**50**/*45*	**50**/*45*	
*Eco*RI	0.6	++	0	**45**/*37*	**50**/*45*	
*Mlu*I	0.6	++	+	**65**/*60*	**70**/*65*	
*Nco*I	0.6	++	+	**60**/*55*	>**62.5**/*60*	
DNaseI	0.6	++	+	**50**/*45*	>**60**/*55*	
RNaseI	0.6	+	+	**50**/*45*	>**60**/*55*	
*Nde*I	0.6	+	+	**55**/nt	**60**/*55*	
*Pvu*II	0.6	+	0	*45*	**50**/*45*	
*Pst*I	0.6	+	0	*37*	**45**/*37*	
*Dra*I	0.6	0	0	*50*	*55*	
*Hin*dIII	0.3	0/+	nt	*65*	*70*	b
*Hin*cII	0.6	0	nt	*37*	*45*	
PNK	0.6	−	−	*37*	*45*	
DNA pol (KF)	0.6	0/−	nt			b
β-Agarase	0.6	0/−	0/−	*55*	*60*	c
T3 RNA pol	0.6	−	−	*37*	*45*	d

Summary of trehalose effect on several enzymes. In the column M, the concentration of trehalose (mol/liter) is shown. In the column stabilization, ++ indicates thermostabilization (5–10°C); +, moderate stabilization (within 5°C); 0/+, possible slight stabilization; 0, no stabilization; 0/−, possible slight inhibition; and −, inhibition. In column Activation: +, activation; 0, no activation; 0/−, possible slight inhibition; −, inhibiton; and nt, not tested. In columns >90% activity and >30% activity, highest temperature tested at which enzymes still showed, respectively, activity that was difficult to distinguish from the maximal (>90%) and still partial activity (>30%). In bold are temperatures relative to enzymatic activity in the presence of trehalose, and in italics are temperatures of enzymes in the absence of trehalose. Temperatures of thermostabilization and thermoactivation are shown only if different from the control reaction. Notes: a, see further characterization; b, reaction in which the enzyme activity was too high before its inactivation thus complicating the detection of differences; c, a shift in the optimum temperature was observed, but an overall decrease in activity was caused by trehalose; and d, trehalose was inhibitory at high concentrations. Data are representative of three or more independent experiments. MMLV, Moloney murine leukemia virus; PNK, polynucleotide kinase; nt; not tested.

tion at 45°C (Fig. 2*A*, lane 1), 5-kb, full length cDNAs also could be synthesized at 55 and 60°C (Fig. 2*A*, lanes 2 and 3), with inactivation only at 65°C (Fig. 2*A*, lane 5). The control reaction at 60°C without trehalose showed complete inactivation of the RT (Fig. 2*A*, lane 4); in this lane, there was no full length cDNA but a smear of cDNA of low molecular weight. The smear corresponds to shortened cDNA that is thought to be synthesized during the step of increasing the temperature, before reaching the incubation condition at 60°C. In fact, the intensity and average length of this smear varied with the speed of the increase in temperature (data not shown).

Our data show that trehalose stimulated RT already at 42°C; in Fig. 2*B*, the yield of full length cDNAs (arrow in Fig. 2*B*) is higher in the presence of trehalose even under the standard reaction temperature (lanes 2, 6, and 10) than in its absence (lanes 1, 5, 9). The partially synthesized products in lanes 1, 5, and 9 (asterisks) were produced in correspondence with the strong nonspecific stopping points that depend on the sequence of mRNA.

When the reaction was performed at 60°C, the yield of full length cDNAs was improved further for the 5- and 5.5-kb cDNAs (Fig. 2*B*, lanes 3 and 7), accompanied by almost complete disappearance of stopping points because of secondary structures. The reaction with the 10-kb of RNA (Fig. 2*B*, lane 11) showed early termination, which may have been caused by inactivation of RT at 60°C before the reaction could be completed perhaps because of some strong RNA secondary structure. Starting from this hypothesis, a temperature cycling program was designed to extend the cDNA at 60°C for 2 min and to renature the RT at 55°C for 2 min. Under these conditions, the full length, 10-kb cDNA became the main reaction product with a sensitive reduction of the nonspecific terminations (Fig. 2*B*, lane 12). The temperature cycling program also gave an excellent yield of full length cDNA for the 5- and 5.5-kb *in vitro* transcripts (Fig. 2*B*, lanes 4 and 8).

Finally, mouse brain mRNA was used as a substrate of thermoactivated RT with the above temperature cycling protocol. In comparison with the classic reaction (Fig. 2*C*, lane 1), the size of the first-strand cDNA synthesized in the presence of trehalose was shifted up in the presence of trehalose even under standard conditions (Fig. 2*C*, lane 2); when cDNA was synthesized with the cycling temperature program in the absence and presence of trehalose, the trehalose-containing reaction performed better, showing the best average length and longest cDNAs, with a remarkable fraction extending far beyond the 9.4-kb marker (arrow), that is, a few kilobases longer than the upper limit of the standard reaction (asterisk). Also, the reaction in Fig. 2*C*, lane 3 (with a cycling program but in the absence of trehalose) showed some long cDNAs. The synthesis of long cDNAs is probably allowed by the long annealing and extension temperature at 45°C and the final extension at high temperature just before the inactivation of RT, which does not allow us to easily discriminate between lane 3 and 4 differences. However, the average cDNA length in Fig. 3, lane 3 is shorter than that of lane 4, in which the thermoactivated RT achieves the highest reaction for a much larger number of cDNAs. Also, by densitometric analysis (not shown), the intensity of the signal was the same, ≈2.2 kb, for lanes 3 and 4, but it decreased between 4.3 and 9.4 kb.

Partial Thermal Stabilization and Activation by Other Sugars. As the final step, we examined the thermoactivation effect of other sugars. Other monosaccharides or disaccharides tested in this study also could induce partial stabilization and/or activation when compared with the effect of trehalose. In Fig. 3, the yield of full length, 5-kb cDNA at 60°C was higher when RT was activated with trehalose (Fig. 3, lane 1) than with sucrose (Fig. 3, lane 2), glucose (Fig. 3, lane 3), and maltose (Fig. 3, lane 4), as confirmed by densitometric analysis. When tested with restriction nuclease *Nco*I and *Eco*RI, glucose gave only marginal stabilization whereas sucrose and maltose were not suitable for restriction digestion because a complex including DNA was formed that inhibited its migration in TBE (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) agarose gel (data not shown).

DISCUSSION

We report enzyme thermostabilization and thermoactivation achieved by the addition of a chaperonin-like molecule to the reaction mixture. Enzyme thermostabilization and thermoactivation pave the way for several new applications in a wide range of fields. In fact, the combination of thermostabilization and thermoactivation may become a very useful tool to reduce the quantity of enzyme and increase the speed of a given reaction. Although conditions may require case by case optimization, the principle and the simplicity of thermoactivation by trehalose addition has great potential to improve cost/performance and time/performance in a very wide range of enzymatic applications, for instance in biochemical reactions, diagnostics, or industrial fields. Even more importantly, thermoactivation can be useful to increase the overall efficiency and extent of the reaction, allowing a yield unexpected under standard condition. Additionally, thermostabilization and thermoactivation can originate new applications, which are impossible at the standard reaction temperature. In fact, several nucleic acids restriction/modification enzymes become thermostable at temperatures suitable, for instance, for high stringency nucleic acid hybridization; this could allow the reaction of only the specifically hybridized nucleic acids. A

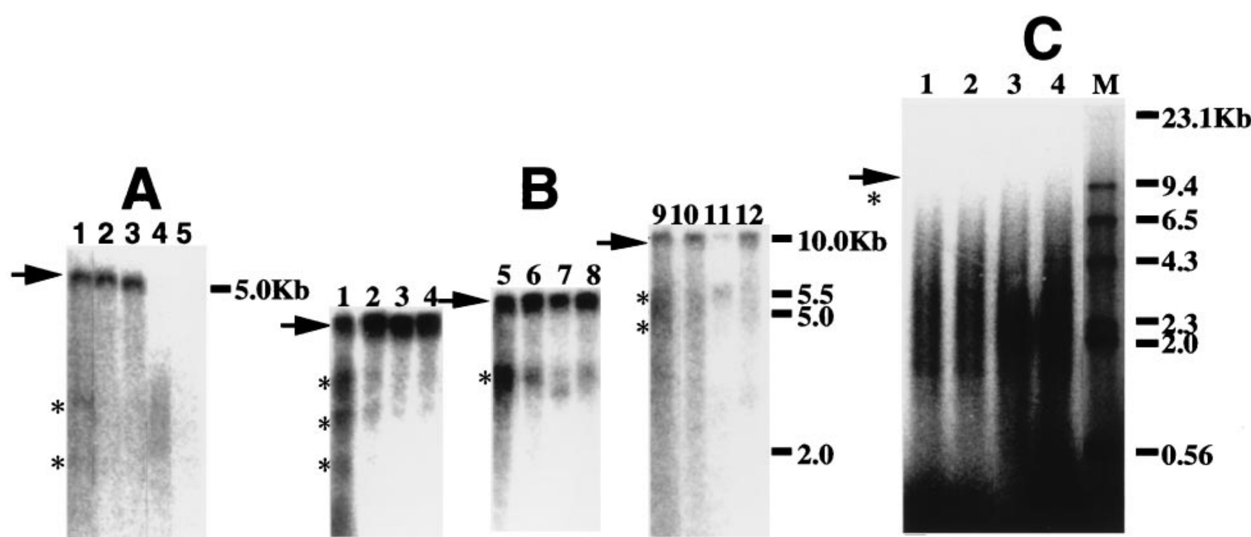

FIG. 2. Improvement of full length cDNA yield by thermostabilization of RT by trehalose. (*A*) 5-kb cDNAs were synthesized in the presence of 0.6 M trehalose at 45°C (lane 1), 55°C (lane 2), 60°C (lane 3), and 65°C (lane 5); lane 4, synthesis at 60°C in the absence of trehalose. (*B*) *In vitro* transcripts were used as templates as follows: lanes 1–4, 5 kb; lanes 5–8: 5.5 kb; lanes 9–12: 10 kb. Lanes 1, 5, and 9: reactions without trehalose; lanes 2–4, 6–8, and 10–12: reactions with trehalose. Lanes 1, 2, 5, 6, 9, and 10: incubation at 45°C; lanes 3, 7, and 11: incubation at 60°C; lanes 4, 8, and 12: incubation with temperature cycling. Arrowheads, full length cDNAs; asterisks, truncated cDNAs. (*C*) RT of mouse brain mRNA: lane 1, standard optimized reaction; lane 2, reaction in the presence of trehalose at standard temperature; lane 3, reaction in absence of trehalose with the temperature-cycling program; lane 4, reaction in presence of trehalose with the temperature cycling program. M, λ-*Hin*dIII markers. Figures are representative of at least three independent experiments. Longer cDNAs synthesized in the presence of trehalose (lane 4, arrow) or under standard best conditions (lane 1, asterisks) are shown.

practical example of a new application is the case of RT reaction. In fact, we demonstrated here the applicability of thermoactivation to drastically improve the final yield of the RT reaction, which was considered until now a low efficiency reaction. RT thermoactivation offers the basis for the preparation of a new generation of full length cDNA libraries (9), which are useful for molecular cloning and for a full length cDNA sequencing approach. Additionally, the range of RT-PCR could be expanded to the amplification of very long mRNAs. Moreover, thermostability of RT implies that higher temperatures can be used to anneal any first-strand cDNA-specific primers, enabling the increase of the priming specificity of RT-PCR.

From our results, we could not rule out whether trehalose could cooperate with other chaperonins. By using other chaperonins in combination with trehalose *in vitro*, we may be able to further expand the temperature range of thermostabilization and thermoactivation. This could further extend the applicability of normally themolabile enzymes as thermostable ones.

Although stabilization of dried enzymes (7) and thermoprotection (6) already was described as a universal property of trehalose, the newly discovered properties of trehalose thermostabilization and thermoactivation are not shared by all of the enzymes that we tested, thus suggesting that some protein motif may be preferentially stabilized by trehalose in aqueous solution. There are two theories to explain the specific stabilization of enzymes in trehalose, which, however, refer to dried enzymes (reviewed in ref. 7). The first is the glass state theory, which says that trehalose solutions undergo glass transformation, resulting in an amorphous continuous phase in which molecular motion and thus degradation are minimized. The second is the water replacement hypothesis, for which polyols (such as sugars) can replace water to make multiple external hydrogen bonds and thus maintain the secondary structure of proteins. Although further studies may help to understand the structural mechanism of this acquired stabilization, we think that there are some additional mechanisms for biomolecule stabilization by trehalose. In fact, the above models refer to the stabilization only in a water-free environment and do not explain the thermostabilization and thermoactivation in aqueous solution. The following two previous observations support the idea that trehalose may stabilize proteins by influencing the protein hydration in solution (12–14). In fact, it has been shown that porcine pancreatic lipase is very active in a water-free organic media at temperatures up to 100°C, but it is inactivated readily in the presence of traces of water (13). Additionally, by studying the denaturation profile in aqueous solution of RNase A (14) and chymotrypsinogen A (15), it has been found that polyols could stabilize these enzymes. This stabilization has been explained to be due to the reduction of the number of hydrogen bonds between protein and water molecules in the presence of polyols, named as the "lessened hydrogen bond-rupturing capacity" of the aqueous medium. Perhaps, trehalose can be viewed as an agent that decreases the

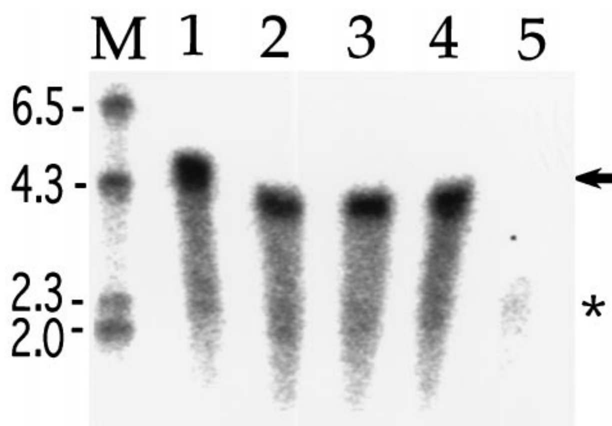

FIG. 3. Comparison of trehalose with other sugars for enzyme stabilization or activation. *In vitro* transcript of 5 kb was used as substrate for cDNA synthesis at 60°C in the presence of the following reaction modifiers: lane 1, trehalose; lane 2, sucrose; lane 3, glucose; lane 4, maltose; lane 5, no addition. Arrow and asterisk, longest full length cDNA detectable in the trehalose-containing reaction and in the absence of trehalose, respectively. Data are representative of more than three independent experiments.

protein hydration in solution and that reduces the hydrogen bond-rupturing capacity of the water but that, in the dried state, additionally can be a water-replacing agent or a glass-like stabilizer. The fact that other monosaccharides and disaccharides [tested previously (6, 7) and in our study] cannot thermoprotect, thermostabilize, and thermoactivate enzymes with the same efficiency indicates that trehalose may have some unidentified structural features that cause a somehow more specific interaction with protein structures.

Is thermoactivation an effect simply due to an increased reaction temperature, allowed by the presence of trehalose or by the direct stimulation of the enzymes by trehalose? Probably both explanations are true, and the main effect may depend on the sort of enzyme. For instance, in Fig. 1*B*, in the case of the restriction enzyme *Nco*I, the increase of activity can be observed by switching the reaction temperature from 37°C to 45°C with and without trehalose. It is suggested that the increased activity in this case is mainly caused by higher heat movement of molecules at 45°C. When further switching the temperature from 45°C to 50°C, the enzyme was inactivated in the absence of trehalose. However, in the presence of trehalose, the enzyme activity continued to increase. This suggests that trehalose can operate, at least in this case, by suppressing the heat denaturation of the enzyme at a higher temperature. On the other hand, in the case of DNaseI, even at 37°C, the optimal reaction temperature, the addition of trehalose activated the enzyme. At 37°C, this activation was not caused by heating; it was an effect of trehalose itself. Also, in the case of DNase I, if the reaction temperature was raised from 37°C to 50°C, a notable increase in enzymatic activity was seen, caused by increased heat movement of the molecules.

Although the presence of trehalose may alter the conformation of nucleic acids, as in the case of the reduction of the RNA secondary structure in the case of RT, we think that the benefits of thermoactivation derive also directly from faster kinetics caused by the increased reaction temperature, sometimes associated with the direct stimulation of enzymes by trehalose. This view is in agreement with the 4-fold increased kinetics of dried lipase at 100°C in organic media (13).

Finally, the possible *in vivo* role of the trehalose-mediated enzyme thermostabilization and thermoactivation (perhaps mediated also by other chaperonins), as observed *in vitro*, is intriguing: it may alter the view of the heat shock response as not only being a cell protection mechanism but also serving an active function in cell homeostasis.

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