Refolding of denatured lactate dehydrogenase by *Escherichia coli* ribosomes

Subrata CHATTOPADHYAY,* Biswadip DAS,* Aloke Kumar BERA,* Dipak DASGUPTA† and Chanchal DASGUPTA*‡

*Department of Biophysics, Molecular Biology and Genetics, University of Calcutta, 92 A.P.C. Road, Calcutta-700009, India, and †Biophysics Division, Saha Institute of Nuclear Physics, 37 Belgachia Road, Calcutta-700037, India

Escherichia coli ribosomes were used to refold denatured lactate dehydrogenase from porcine muscle. This activity of ribosomes, unlike most of the chaperons, did not require the presence of ATP. The molar concentration of ribosomes required for this refolding was comparable with that of the enzyme. Restoration of the enzyme activity was demonstrated using assays for both the forward and backward reactions. Binding of the denatured enzyme to ribosomes and its refolding were fairly rapid processes as revealed by the time course of the reaction and inhibition of folding when the denatured enzyme was allowed to refold spontaneously for short times before the addition of ribosomes. This protein-folding activity was detected in 70 S ribosomes as well as its RNA, in 50 S particles and in 23 S rRNA. However, 30 S particles failed to refold the enzyme.

INTRODUCTION

The mechanism of folding of polypeptide chains into three-dimensional active proteins remains an enigma. Two important considerations led us to design experiments to check whether ribosomes could play a direct role in the process of folding a polypeptide chain into the three-dimensional active conformation. (1) Polypeptides are synthesized on ribosomes which are large complex ribonucleoprotein structures that might have such post-translational activity and (2) there have been reports on *in vitro* synthesis of a number of proteins by ribosome-containing extracts from cells, where the proteins were found to possess biochemical activity [1,2]. Some proteins have been found to be folded on ribosomes [3]. Of course, these findings do not rule out the possibility of chaperons taking part in such processes in association with ribosomes.

In the course of our studies, we found that ribosomes from a number of sources, such as *Escherichia coli*, wheat germ and rat liver, could fold a number of proteins from a variety of sources [4]. It appeared that protein-folding activity of ribosomes is a general one. We then started a series of systematic studies on the folding of a number of proteins. In the present paper, we report our findings on the folding of lactate dehydrogenase from porcine muscle with the help of *E. coli* ribosomes.

We also tried to locate this activity on the ribosomes. The 70 S and 50 S particles, the total rRNA and 23 S rRNA were all found to possess this activity. The 30 S particles contributed no more than a small non-specific protection similar to that shown by BSA.

MATERIALS AND METHODS

Preparation of *E. coli* 70 S ribosomes and their subunits

70 S ribosomes were purified from *E. coli* MRE 600 cells by the method of Nomura and Watson [5]. The high-salt-washed (1 M NH_4Cl) ribosomes were purified through 5–20% sucrose gradients in 20 mM Tris/HCl, pH 7.5, containing 10 mM magnesium acetate, 30 mM NH_4Cl and 1 mM dithiothreitol and stored at −70 °C.

The 50 S and 30 S subunits were purified by dialysing 70 S ribosomes against 20 mM Tris/HCl, pH 7.5, containing 30 mM NH_4Cl and 0.1 mM magnesium acetate, followed by 5–20% sucrose-gradient sedimentation in the same buffer. To avoid cross-contamination, fractions from the heavier side of the 50 S peak and lighter side of the 30 S peak were pooled and precipitated with chilled ethanol and kept at −70 °C until use.

Preparation of rRNAs

Total rRNA and 23 S rRNA were isolated from *E. coli* 70 S and 50 S ribosomal particles respectively by extracting these eight times with phenol [6]. The 23 S rRNA was separated from 5 S rRNA by gel-filtration chromatography. The purity of the 23 S rRNA was ensured by the following criteria: it (a) gave a single band on 2% agarose–formaldehyde gel [7], (b) had a A_{260}/A_{280} ratio of 2.1, (c) showed 40% hyperchromicity after RNAase digestion at 37 °C, and (d) exhibited no detectable fluorescence emission when excited at 285 nm and 292 nm at a concentration of 300 μg/ml.

Denaturation of the enzyme

Porcine muscle lactate dehydrogenase was obtained from SRL India. The specific activity of the enzyme was 800 μmol/min per mg, and 5 μg of the enzyme gave a single band on SDS/PAGE with silver staining (results not shown). It was diluted in 20 mM Tris/HCl, pH 7.5, to obtain a working concentration of 1 mg/ml, and denatured with 6 M guanidinium chloride (Sigma) in the presence of 5 mM 2-mercaptoethanol for 30 min at 37 °C. If assayed at this point immediately after dilution in reaction buffer, the residual activity is less than 1%, but it increases slightly with time if the enzyme is allowed to fold spontaneously as shown in Figure 1.

Refolding of the denatured enzyme

The denatured enzyme was diluted 40-fold in buffer C (20 mM Tris/HCl, pH 7.5, 10 mM magnesium acetate and 5 mM 2-mercaptoethanol) containing any one of the following: *E. coli* 70 S, 50 S, 30 S particles, total rRNA or 23 S rRNA. Incubation was for 30 min at 37 °C unless otherwise stated.

Enzyme assay

The re-activated enzyme was diluted about 160 times in the assay mixture. The forward reaction ($NADH \rightarrow NAD^+$) mixture

‡ To whom correspondence should be addressed.

contained 100 mM Tris/HCl, pH 7.5, 5 mM sodium pyruvate, 250 μM NADH [8] and the enzyme at 37 °C. The decrease in absorbance/min was measured at 340 nm. The assay mixture for the backward reaction ($NAD^+ \rightarrow NADH$) contained 500 mM Tris/HCl, pH 9.0, 250 mM sodium lactate, 250 μM NAD^+ [9] and the enzyme. The reaction volume was 1 ml in each case. The increase in absorbance/min at 37 °C was measured at 340 nm. The percentage recovery of forward and reverse activities of the enzyme were calculated by comparing the initial rates of these two reactions with those of equivalent amounts of native enzyme. The forward and reverse reaction rates, defined as above, were different for the native enzyme. The specific activity of refolded enzyme at optimum level of recovery was about 400 μmol/min per mg.

Each experimental point on the curves shown in the Results section is the mean ± S.E.M. of three or more independent experiments.

RESULTS

Time course for recovery of enzyme activity with 70 S ribosomes from *E. coli*

From the denaturation mixture at 30 min, the enzyme was diluted in buffer C, containing *E. coli* 70 S ribosomes, the final concentration of enzyme tetramer and 70 S ribosomes being 0.1 and 0.05 μM respectively. The mixture was incubated at 37 °C for various times, and the time course of recovery of enzyme activity was followed by measuring the velocities for both the forward and backward reactions. As shown in Figure 1, the recovery of enzyme activity was linear with time for 10 min. Although the rates of these two reactions were different, the maximum recoveries of both forward and reverse activities were the same (about 50%). We found this by comparing the two rates with those of equivalent amounts of native enzyme. It should be noted that the two reaction rates were also different for native enzyme. ATP, GTP and UTP had no effect on the extent of recovery of enzyme activity (results not shown).

The recovery of enzyme activity in the absence of ribosomes was about 5%. Refolding of denatured lactate dehydrogenase spontaneously on removal of denaturing agent has been reported by Rudolph et al. [10]. Although their conditions of denaturation and refolding were different from ours, they obtained similar levels of recovery (about 5%) of enzyme activity after 30 min of refolding, when the residual guanidinium chloride concentration was 0.12 M. In our experiments, the guanidinium chloride concentration was 0.15 M. Only with the lower residual guanidinium chloride concentration did they obtain a higher level of recovery of the activity.

Dependence of recovery of enzyme activity on the concentration of ribosomes

The recovery of enzyme activity increased with the concentration of ribosomes up to a ratio of ribosome to enzyme tetramer of 1:1, but did not increase further with further addition of ribosomes (Figure 2). The maximum recovery of both forward and reverse activities was about 40–50% of native enzyme activity in this case. BSA, at a much higher concentration than that of the ribosomes, increases the recovery of enzyme activity by about 4% over the spontaneous recovery (Figure 2). BSA was added as a possible non-specific protector of labile folding intermediates, as one could argue that the ribosome-mediated recovery of enzyme activity is a non-specific effect. Figure 2 also shows the recovery of enzyme activity with 80 S ribosomes from rat liver. The maximum level of recovery was about 60%. Detailed results of the recovery of enzyme activity with the subunits and rRNA from 80 S ribosomes will be given elsewhere.

Effect of temperature on the recovery of enzyme activity

In this experiment, lactate dehydrogenase was denatured at 37 °C and subsequently incubated at various temperatures with and without ribosomes. Finally the assay for the refolded enzyme was carried out at 37 °C, the optimum temperature for the activity of the native enzyme. Maximum recovery of both forward and reverse activities was seen when the enzyme was incubated with or without ribosomes at 37 °C (Figure 3). The recoveries of

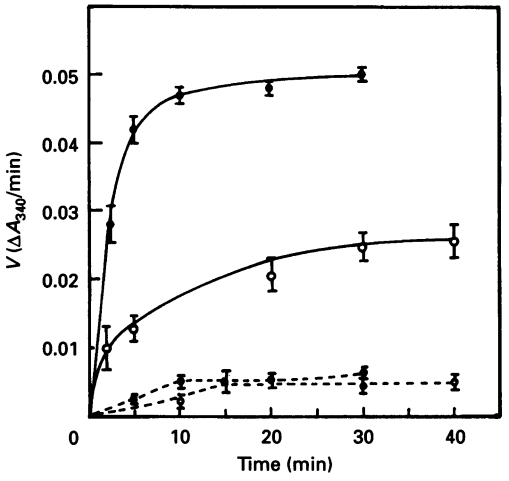

Figure 1 Time course of re-activation of denatured lactate dehydrogenase in the presence and absence of ribosomes

Forward activity with (●——●) and without (●- - - -●) ribosomes. Backward activity with (○——○) and without (○- - - -○) ribosomes.

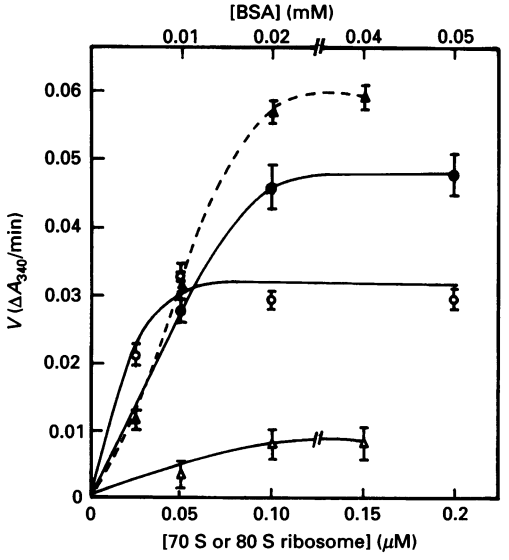

Figure 2 Effect of concentration of 70 S ribosomes on the recovery of the forward (●) and backward (○) activities of denatured lactate dehydrogenase

△, Effect of BSA on the recovery of forward activity; ▲, effect of rat liver 80 S ribosomes on the recovery of denatured lactate dehydrogenase activity.

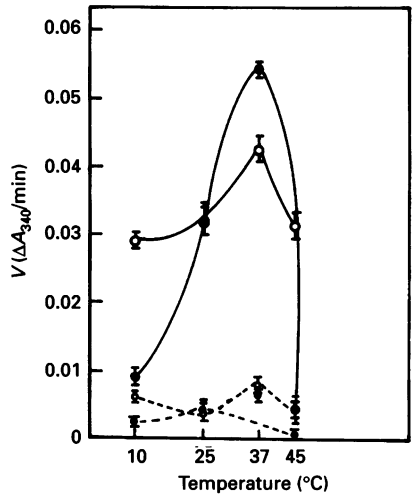

Figure 3 Temperature-dependence of re-activation of denatured lactate dehydrogenase incubated in the presence and absence of ribosomes

Note that the enzyme was assayed at 37 °C. Forward activity with (●——●) and without (●----●) ribosomes. Backward activity with (○——○) and without (○----○) ribosomes.

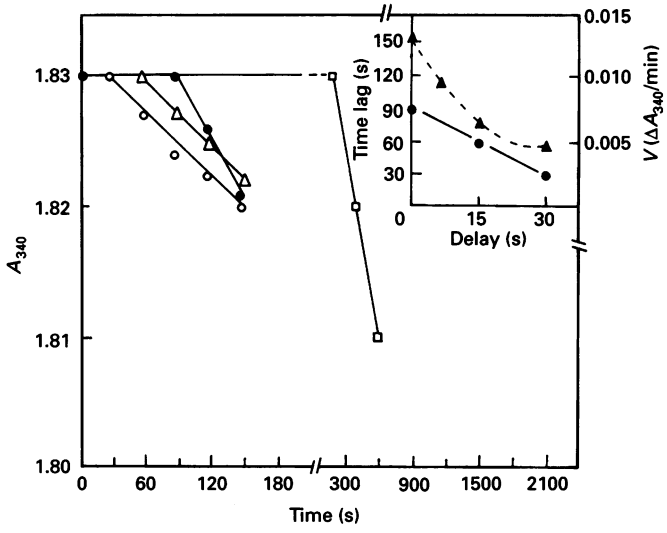

Figure 4 Effect of delay in addition of ribosomes on the *in situ* refolding reaction

The open boxes (□) show poor recovery of enzyme activity in the absence of ribosomes. The process of recovery started very slowly after 300 s (note the expanded scale). The recovery of enzyme activity started after 90 s, 60 s and 30 s when the delay in adding the ribosomes was 0 s (●), 15 s (△) and 30 s (○) respectively. The inset shows the effect of delay in adding ribosomes on the lag in the onset (●) and extent (▲) of recovery of enzyme activity. Note that the lag in the onset as well as the extent of recovery of enzyme activity decreased with increasing delay.

the forward and backward activity of the enzyme in the presence and absence of ribosomes were around 60 % and 5 % respectively.

Effect of delay in adding ribosomes to the folding reaction

In one set of experiments, the ribosomes and substrates for the forward reaction, e.g. sodium pyruvate and NADH, were all added to the denatured enzyme at the same time. In the final

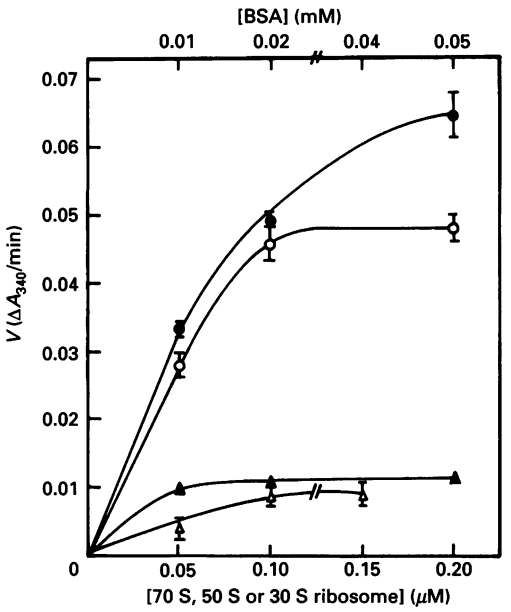

Figure 5 Effect of concentration of 70 S (○), 50 S (●) and 30 S (▲) particles on the recovery of the forward activity of denatured lactate dehydrogenase

△, Effect of BSA on the recovery of the forward activity.

reaction volume, the concentration of denatured enzyme (considering tetrameric molecular mass) and ribosomes were 0.01 μM and 0.1 μM respectively. The amount of enzyme was very low compared with the concentration of ribosomes (see the Discussion section). The activity of the enzyme was monitored by the fall in A_{340}. The reaction started after a lag of about 90 s (Figure 4), which marked the minimum time required to initiate the ribosome-mediated refolding of the enzyme. In other sets of experiments, ribosomes were added to the reaction mixture at later times. As a result, the time lags for onset of reactions were reduced and so were the extents of the recovery of enzyme activity (inset in Figure 4). The ribosomes were therefore delaying the time for the collapse of the denatured enzyme, probably giving the polypeptide a chance to take up the active conformation (see the Discussion section). In the absence of ribosomes, the self-folding process was poor and took longer than ribosome-mediated folding (Figure 4). These experiments could not be performed for the reverse activity of the enzyme because the pH optimum for this reaction was 9.0, whereas the refolding experiment was performed at pH 7.5.

Recovery of enzyme activity in the presence of 50 S particles, total rRNA and 23 S rRNA

Figure 5 shows the recovery of enzyme activity in the presence of 70 S, 50 S and 30 S particles of *E. coli*. In this experiment the recovery of enzyme activity was found to be higher with 50 S than with 70 S ribosomes, whereas the recovery with 30 S particles was the same as with BSA.

As 70 S and 50 S particles both showed protein-folding activity, we wanted to see whether their RNA molecules would show similar activity. In fact, Noller et al. [11] demonstrated peptidyltransferase activity in the 23 S rRNA of the 50 S subunit. Total rRNA and 23 S rRNA were found to have considerable protein-folding activity (Figure 6), with maximum activity recovery of 52 % and 35 % respectively. The activity of total rRNA

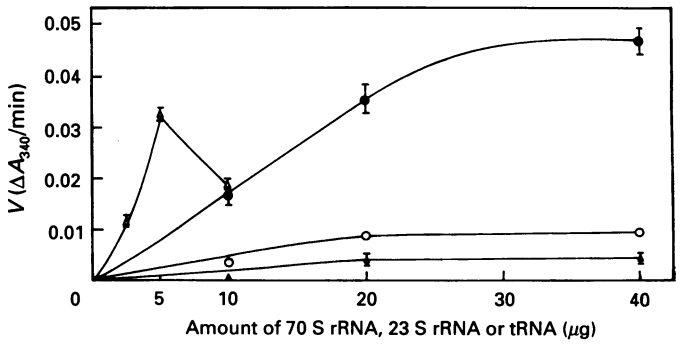

Figure 6 Effect of total rRNA (●), 23 S rRNA (△) and tRNA (○) of *E. coli* on the recovery of denatured lactate dehydrogenase activity

▲, Effect of total rRNA, pretreated with RNAase A (100 μg/ml) at 37 °C for 45 min and then for 30 min at 65 °C. The reaction volume was 200 μl in each case.

was destroyed when it was pretreated with RNAase A (100 μg/ml). *E. coli* tRNA, which has considerable secondary structure, could not refold the enzyme.

Maximum recovery of enzyme activity was obtained at a considerably lower concentration of 23 S rRNA than total rRNA. In fact, with higher concentrations of 23 S rRNA, the recovery of enzyme activity decreased (Figure 6); we observed this consistently in a number of experiments. Further experiments need to be carried out to obtain an explanation for this fall in recovery with increasing 23 S rRNA concentration.

DISCUSSION

Lactate dehydrogenase from pig muscle is a tetrameric enzyme. The monomer has five cysteines although none of them is involved in disulphide bridge formation [9]. Therefore the principal interactions during denaturation and refolding of this enzyme would be breaking and making of salt bridges, hydrogen-bonding and hydrophobic interactions. The enzyme did not fold significantly by itself in the time in which it was folded by the ribosomes or rRNA. The forward and reverse activities of LDH were recovered to the same extent with the help of 70 S ribosomes. This suggests that the enzyme might have regained its native form by ribosome-mediated folding.

As the GroEL proteins of *E. coli*, chaperons that can cause folding of many proteins, have been reported to be associated with ribosomes under some conditions, we checked our ribosome preparation for possible GroEL contamination by immunodot blot with polyclonal antibodies against Hsp60 (kindly provided by Professor S. C. Lakhotia, Benaras Hindu University, Varanasi, India). Whereas the immunoprecipitation was very strong against *E. coli* extract containing GroEL proteins, almost no precipitation was visible for 70 S and 50 S ribosomal particles (results not shown). Also immunoprecipitation of 10 ng of *E. coli* GroEL protein and 1 μg each of 70 S and 50 S ribosomes was carried out with antibody against GroEL using e.l.i.s.a. Whereas strong precipitation was obtained against GroEL protein, the signals from 70 S ribosomes were negligible and no immunoprecipitation at all was obtained in the case of 50 S particles (results not shown). Therefore the refolding activity of ribosomes was not due to GroEL contamination. Also, unlike for GroEL proteins, ATP was not required for ribosome-mediated protein folding.

As we see from Figure 4, spontaneous folding of the enzyme was the slowest process and recovery of activity was very poor. Ribosome-aided folding was maximum when the denatured enzyme was diluted straight into ribosome-containing buffer. The process was faster than spontaneous folding, but it was even more rapid if the delay in adding the ribosomes was increased. The recovery of activity, however, was less with delayed addition of ribosomes. After a 30 s delay, the recovery was as low as that in the case of spontaneous folding.

We propose that ribosomes are involved at two stages of the folding process. The first stage is slow, as ribosomes regulate the hydrophobic collapse of polypeptides by preventing counterproductive intramolecular hydrophobic interactions, thereby giving them temporal and spatial advantage to fold in a more co-ordinated fashion. In the second stage, they guide the rest of the polypeptide to fold properly by a fast reaction to reconstitute the active sites, etc.

Looking at the temperature profiles of protein-folding by ribosomes for lactate dehydrogenase and all other proteins studied so far, we found that the optimum temperature for folding coincided with the optimum temperature for activity of each protein. For those proteins that refolded spontaneously to a detectable level, the optimum temperature for spontaneous folding was also the same. As the conformations attained spontaneously by the native proteins at optimum temperatures were the best for their activity, we think that, after the regulated hydrophobic collapse, ribosomes also prevented unwanted fluctuation in the polypeptides around their active sites. This second most important step exhibited the typical single temperature-dependence for ribosome-mediated folding, spontaneous folding and enzyme activity, for all the enzymes that we have studied so far [4] (S. Chattopadhyay, B. Das, A. K. Bera and C. Dasgupta, unpublished work).

In our experiments, increasing ribosome concentrations did not increase the yield of re-activated enzyme beyond the plateau, which was in the range 40–50%. This could be due to several factors. Increasing the ribosome concentration could lead to their self-aggregation and loss of accessibility of the active sites to the enzyme. An increase in light scattering and visible precipitation took place at very high ribosome concentrations. Several other factors, such as the presence of cofactors, hydrophobicity of the medium, ionic strength, concentration of glycerol etc., often control the extent of enzyme re-activation. These parameters need looking at more carefully.

We have observed folding of denatured lactate dehydrogenase with 70 S ribosomes and 50 S particles, but not with 30 S particles. Denatured horseradish peroxidase and bacterial alkaline phosphatase could also be re-activated by 70 S and 50 S ribosomal particles but not by 30 S particles (S. Chattopadhyay, B. Das, A. K. Bera and C. Dasgupta, unpublished work). We therefore think that the 70 S and 50 S particles have general protein-folding properties. Total rRNA and 23 S rRNA from these particles were also found to possess protein-folding activity. As newly synthesized polypeptides leave ribosomes via the exit on the 50 S subunit, the protein-folding activity could very well take place there and in the major rRNA of the 50 S subunit. In this context, we recall the report on the presence of peptidyltranferase activity on 23 S rRNA [11].

We gratefully acknowledge Professor A. Lahiri Majumdar (Bose Institute, Calcutta, India) for providing spectroscopic facilities. This work was supported by CSIR grant no. 9/358/91 EMR-II and BRNS grant no. 4/25/92/GG. S.C. is a UGC Junior Research Fellow and B.D. and A.K.B. are CSIR Senior Research Fellows.

REFERENCES

1 Zubay, G., Chambers, D. A. and Cheong, L. C. (1970) in The Lactose Operon, p. 375, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

2 deCrombrugghe, B., Chen, B., Anderson, W., Gottesman, M., Pastan, I. and Perlman, R. (1971) Nature (London) New Biol. **231**, 139
3 Fedorov, A. N., Friguet, B., Djavadi-Ohaniance, L., Alakhov, Y. B. and Goldberg, M. E. (1992) J. Mol. Biol. **228**, 351–358
4 Das, B., Chattopadhyay, S. and DasGupta, C. (1992) Biochem. Biophys. Res. Commun. **183**, 774–780
5 Nomura, M. and Watson, J. D. (1959) J. Mol. Biol. **1**, 204–217
6 Amils, R., Mathew, E. A. and Cantor, C. R. (1978) Nucleic Acids Res. **5**, 2455–2470
7 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., pp. 7.43–7.45, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
8 Badcoe, I. G., Smith, C. J., Wood, S., Halsall, D. J., Holbrook, J. J., Lund, P. and Clarke, A. R. (1991) Biochemistry **30**, 9195–9200
9 Boyer, P. D. (1975) Enzymes **11**, 199–202
10 Rudolph, R., Zettlmeisl, G. and Jaenicke, R. (1979) Biochemistry **18**, 5572–5575
11 Noller, H. F., Hoffarth, V. and Zimniak, L. (1992) Science **256**, 1416–1424

Received 24 August 1993/21 December 1993; accepted 21 January 1994