A MICROCALORIMETRIC STUDY ON THE UNFOLDING OF *E. COLI* RIBOSOME

Kin-Ping WONG*, John M. DUNN, and Jesse S. BINFORD, Jr.
Department of Chemistry, University of South Florida
Tampa, Florida 33620, USA

Received 4 March 1974

1. Introduction

The successful in vitro reconstitution of functional ribosome solely from its biomolecular component proteins and RNA's [1] suggested that the information required for the assembly of a ribosome is encoded in the sequence of the protein and the of the RNA's. It appears likely that under proper conditions the proteins and the RNA's are folded into their corresponding three-dimensional structure directed by their sequence. The unique conformation of these biomolecules then enable them to undergo various types of protein–protein and protein–RNA interactions to assemble into a unique functional three-dimensional structure presumably through successive conformational changes. In an attempt to understand the biomolecular mechanism of this assembly, we have undertaken the structural, thermodynamic, and kinetic studies on the unfolding and refolding of ribosome. We have chosen for this study the major unfolding reaction of ribosome by removal of magnesium ion [2–6] since the unfolded inactive ribonucleoprotein complexes have been reported by these investigators to retain all or most of their ribosomal components, and the observed thermodynamic quantities will reflect mainly the conformational changes involved.

In this communication we report the preliminary results on the thermodynamic measurements of the major unfolding of ribosome by microcalorimetry. A single endothermic fast reaction with an enthalpy change: $\Delta H^{I}_{25^\circ C} = +8{,}450$ kcal/mole is observed at 25°C. However, two separate reactions occur sequentially at 37°C. The first reaction which is fast is similar to the one observed at 25°C, and has an enthalpy change $\Delta H^{I}_{37^\circ C} = +11{,}740$ kcal/mole; whereas the second reaction can be observed about 15 min after the initial incubation at 37°C and has a $\Delta H^{II}_{37^\circ C} = +38{,}408$ kcal/mole. At 25°C we have been unable to observe any reaction corresponding to the second reaction within the experimental time which is approximately 3 hr. The change of heat capacity for the fast reaction, ΔC^{I}_{p}, has been estimated to be 274 kcal/deg. mole.

2. Materials and methods

2.1. *Purification of ribosome*

E. Coli MRE 600 cells were grown under forced aeration at 37°C in a Tryptone broth (1.3% tryptone 0.7% NaCl) at pH 7.0. The cell suspension was poured over crushed ice and immediately centrifuged in a Sharpless continuous flow centrifuge. The cell paste was frozen and stored at −70°C. Ribosome is prepared according to the procedure of Nomura and coworkers [7] with slight modifications. The frozen cell paste was thawed in TMA I buffer (Tris–Cl, 0.1 M; NH_4Cl, 3×10^{-2} M; $MgCl_2$, 10^{-2} M; pH 7.2) for 24 hr. The cream was then disrupted in a French press at 9 to 12×10^3 psi.

2.2. *Flow microcalorimetry*

Calorimetric measurements were carried out using a flow modification of the Beckman model 190 micro-

* Public Health Service Research Career Development Awardee (GM 70628), to whom all correspondence should be addressed.

calorimeter [8, 9]. The ribosome solution in 1 mM Tris–Cl buffer at pH 7.2 was introduced into the calorimeter simultaneously with an appropriate concentration of EDTA solution in 1 mM Tris–Cl buffer at pH 7.2 via 5 ml Hamilton glass-tip syringes. The molar ratio of EDTA to ribosome is maintained at 3.252×10^3. At this concentration ratio maximum unfolding of the ribosome is attained according to Tal [6]. Approximately 7 minutes of flow time was required after flow was initiated in the calorimeter to establish a steady-state which could be used to determine the rate of heat output. The reaction was monitored at various flow rates and concentrations. The slower reaction was determined by stopping the calorimetric flow about 15 minutes after the steady state condition had been established. After a return to the baseline, time was allowed for a second endothermic response to occur. Calibration of the calorimeter was done with the NaOH–HCl neutralization reaction. Carbon dioxide-free NaOH solution was used. The heat of neutralization for this reaction is 13.37 kcal/mole.

2.3. *Miscellaneous*

Ribosome concentration was determined by ultraviolet absorption at 260 nm using a Cary 14 spectrophotometer. A molecular weight of 2.65×10^6 g/mole and an extinction coefficient of $A^{1\ mg/ml}_{260\ nm} = 15.5$ were used. All pH measurements were done with a Radiometer Model 26 pH-Meter with combined glass electrode.

3. Results

3.1. *Flow microcalorimetry*

Fig. 1 presents a typical thermogram of ribosome unfolding traced from the original chart paper. It is clear that a fast endothermic reaction occurs within seconds after mixing ribosome with EDTA at both 25°C and at 37°C as shown in fig. 1(a). The calorimeter flow is then stopped and allowed to return to the zero thermopile output baseline indicating no further reaction involving heat. Completeness of this fast reaction was also confirmed by measurements at other flow-rates varying from 0.311 ml/min. to 0.081 ml/min. There is no further heat response at 25°C through a period of about 3 hr. However, at 37°C a second endothermic reaction begins to appear about 15 min after the flow was stopped and the thermopile output returned to zero baseline. This second reaction

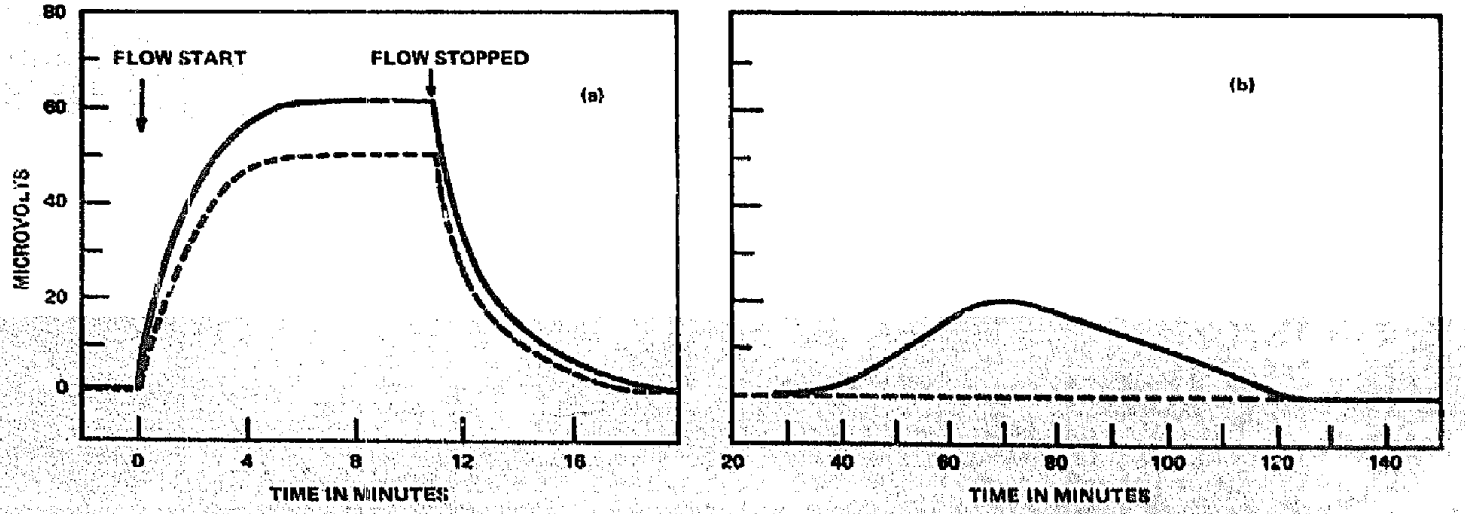

Fig. 1. A typical thermogram of the endothermic heat effect upon the mixing of ribosome with EDTA at pH 7.2, both in 1 mM Tris–Cl. The traces of the curves show the amplified thermopile output, recorded at 100 μV sensitivity. The reaction is measured at 37°C with a ribosome concentration of 1.34 mg/ml (solid curve), and at 25°C with a ribosome concentration of 1.78 mg/ml (dotted curve). The total flow rate in both experiments is 0.158 ml/min. On the actual thermogram there is an auxiliary trace. This trace is the output of a ball-and-disc integrator which gives the area under the thermopile output curve. Note the difference in time scale between fig. 1(a) and fig. 1(b).

continued and was completed after approximately 150 min as shown in fig. 1(b).

3.2. *The fast reaction of unfolding*

The results of calorimetric measurements for the spontaneous unfolding reaction at 25°C are presented in table 1. Each of the experiments presented is an average of triplicate runs with an average deviation of ± 0.6%. Flow rate was varied to ascertain the completeness of the reaction. The other variable is ribosome concentration. Two dilution experiments were performed. The heat of dilution of ribosome is approximately 20 kcal/mole, and that of EDTA is negligible. Both of these heat effects were insignificant compared to the heat effect of the unfolding reaction. The experimental enthalpy change, ΔH_{expt}, is the parameter calculated directly from the calorimetric data and corresponds to the reaction: Ribosome-Mg^{+2} (Expanded) + LH^{-3} + B ⇄ Ribosome (Unfolded) + LMg^{-2} + HB^{+}, where LH^{-3} is EDTA at pH 7.2 and B^{-} is the Tris—Cl buffer. To obtain the enthalpy changes due to unfolding of the ribosome, $\Delta H_{unfolding}$, the heat involved in chelation of Mg^{+2}, ΔH_1, and the heat of proton ionization of EDTA, ΔH_2, and the heat of protonation of the Tris—Cl buffer must be accounted for. These heat contributions have been reported and are listed together with their corresponding reactions as follows:

$$L^{-4} + Mg^{+2}\,(aq) \rightleftarrows L\,Mg^{-2} \qquad \Delta H_1 = +\ 3.49 \text{ kcal/mole} \quad [10]$$

$$LH^{-3} \rightleftarrows L^{-4} + H^{+} \qquad \Delta H_2 = +\ 4.35 \text{ kcal/mole} \quad [11]$$

$$H^{+} + B^{-} \rightleftarrows HB \qquad \Delta H_3 = -\ 11.43 \text{ kcal/mole} \quad [12]$$

Thus, the $\Delta H_{unfolding} = \Delta H_{expt} - 1000\,(\Delta H_1 + \Delta H_2 + \Delta H_3)$. The factor 1000 is introduced to account for the fact that approximately 1000 atoms of Mg^{+2} are bound to each "expanded ribosome particle" [13]. From the values of $\Delta H_{unfolding}$ given in table 1, an average value of + 8,448 kcal/mole was obtained at 25°C and + 11,740 kcal/mole at 37°C. From these two values of $\Delta H_{unfolding}$, the heat capacity change at constant pressure of the fast reaction of ribosome unfolding, ΔC^{I}_{p}, is estimated to be 274 kcal. $deg.^{-1}$ $mole^{-1}$.

3.3. *The slow second reaction of unfolding*

Table 2 summarizes the results on the enthalpy change of the slow second reaction involved in ribosome unfolding. The amount of endothermic heat involved in this slow reaction was measured and reported as $\Delta H_{observed}$ without any correction. In Experiment 1 of Table 2, no heat effect was observed at 25°C during the time period of the calorimetric measurement which is about 3 hr. The amount of endothermic heat involved in this slow temperature-stimulated reaction after rapid mixing of ribosome and EDTA was calculated as shown under Experiment 2a, 2b, and 2c

Table 1
Results of the calorimetric measurements on the fast reaction of ribosome unfolding at 25°C and 37°C; pH 7.2

Exp.	Ribosome conc. (mg/ml)	EDTA conc. (mM)	Total flow rate (ml/min)	Temp. °C	$\Delta H^{I}_{Exp.}$ kcal/mole	$\Delta H^{I}_{unfolding}$ kcal/mole
1	1.79	2.2	0.113	25.0	+5230	+7950
2a	1.79	2.2	0.158	25.0	+5850	+8570
2b	1.79	2.2	0.158	25.0	+5850	+8570
2c	1.79	2.2	0.158	25.0	+5750	+8470
3a	1.79	2.2	0.081	25.0	+5890	+8610
3b	1.79	2.2	0.081	25.0	+5800	+8520
4a	0.64	0.78	0.311	37.0	+9440	+12 210
4b	0.64	0.78	0.311	37.0	+9660	+12 480
5	1.39	1.65	0.311	37.0	+8470	+11 290
6a	1.39	1.65	0.158	37.0	+8660	+11 380
6b	1.39	1.65	0.158	37.0	+8610	+11 330

Table 2
The enthalpy change of the slow second reaction in the unfolding of ribosome at pH 7.2

Exp.	Conditions	Calorimeter temp. °C	Ribosome conc. (mg/ml)	EDTA conc. (mM)	Total flow rate, ml/min	ΔH^{II}_{obs} kcal/mole/deg
1	No incubation period for ribosome with EDTA. Effect is observed after the fast reaction	25.0	1.79	2.2	0.159	0
2a	Same as Exp. 1	37.0	1.30	1.60	2.4	+40 385
b	Same as Exp. 1	37.0	1.30	1.60	2.4	+37 626
c	Same as Exp. 1	37.0	0.93	1.15	2.4	+37 213
3	Incubation of ribosome with EDTA at 25°C for 1.5 hr	37.0	0.84	1.04	2.4	+40 000

in table 2. In these experiments the flow rates were increased drastically in order to deliver the mixture into the calorimeter as quickly as possible so that the flow could be stopped after about one minute. The viscous heat produced during the rapid flow was dissipated in less than 10 min, and the slow reaction could be observed after a return to the baseline. Essentially the same results were obtained in all three cases. An average value of 38,408 kcal/mole is obtained for this slow second reaction. In Experiment 3, the ribosomes were premixed and incubated with EDTA at 25°C for 1.5 hr before they are injected into the calorimeter at 37°C and measured. This was to insure completion of the fast reaction. Under such conditions the second reaction was still observed with $\Delta H^{II}_{obs} = +40{,}000$ kcal/mole. It further confirms that the slow second reaction does not occur at 25°C in a length of time sufficient to be observed at 37°C. If it does it will appear at a later time.

4. Discussions

The unfolding of ribosome by removal of magnesium ions was reported earlier to be irreversible under the experimental conditions [4, 6]. However, recent studies by Traub and Nomura [14] have shown that refolding of the 30 S subunit can be accomplished if it is done under reconstitution condition. The reversibility of the unfolding process thus allows us to interpret the large endothermic enthalpy change of unfolding to correspond to a large exothermic enthalpy change of refolding. This large exothermic enthalpy change of refolding is probably the important factor contributing to the driving force for the assembly of the biomolecular components into a functionally active ribosome which is highly organized and compact. This large exothermic enthalpy change is probably required to compensate for the extremely unfavorable entropy change of assembly.

The slow second reaction of unfolding which can only be observed readily at 37°C and has a large enthalpy change may be significant in regard to the critical temperature conditions necessary for the reconstitution of active ribosomes [14] and may also explain why earlier attempts to refold the ribosome by adding magnesium ion at 25°C have failed, whereas reversibility is achieved at reconstitution condition.

The anomalous large positive value of heat capacity change, ΔC^{I}_{p}, which accompanies the fast reaction of unfolding, may reflect conformational changes which involves the exposure of hydrophobic region of the ribosome to an aqueous environment. Such anomalous positive heat capacity change has been found for the exposure of hydrophobic moiety of organic molecules to water [15, 16] and the unfolding of globular pro-

teins which expose the hydrophobic amino acid residue to an aqueous environment [17].

Microcalorimetric techniques are applicable to the direct thermodynamic study of structural changes of ribosome and provide another approach to investigate the nature of the assembly process. The structural studies of these reactions on ribosome unfolding have been investigated by circular dichroism and will be reported elsewhere. Similar thermodynamic and structural studies on individual subunits are in progress.

Acknowledgements

The financial support of the Damon Runyon Fund (DRG—1165) is gratefully acknowledged.

References

[1] Nomura, M. (1973) Science 179, 864–873.
[2] Spirin, A. S., Kisselev, N. A., Shukulov, R. S. and Bogdanov, A. A. (1963) Biokhimya 28, 920–930.
[3] Cammack, K. A. and Wade, H. E. (1965) Biochem. J. 96, 671–680.
[4] Gesteland, R. F. (1966) J. Mol. Biol. 18, 356–371.
[5] Gavrilova, L. P., Ivanov, D. A. and Spirin, A. S. (1966) J. Mol. Biol. 16, 473–489.
[6] Tal, M. (1969) Biochim. Biophys. Acta 195, 76–86.
[7] Traub, P., Mizushima, S., Lowry, C. U. and Nomura, M. (1971) Meth. Enzymol. 20, 391–407.
[8] Sturtevant, J. M. and Lyons, P. A. (1969) J. Chem. Thermodynamics 1, 201–209.
[9] Velick, S. F., Baggot, J. P. and Sturtevant, J. M. (1971) Biochemistry 10, 779–786.
[10] Anderegg, G. (1963) Helv. Chim. Acta 47, 1801–1814.
[11] Christensen, J. J., Izatt, R. M., Wrathall, D. D. and Hansen, L. D. (1969) J. Chem. Soc. A 1212–1223.
[12] Woodhead, M., Paabo, M., Robinson, R. A. and Bates, R. G. (1965) J. Research Nat'l. Bur. Std. 69A, 263–270.
[13] Spirin, A. S. (1969) Prog. Biophys. Mol. Biol. 19, 135–174.
[14] Traub, P. and Nomura, M. (1969) J. Mol. Biol. 40, 391–413.
[15] Edsall, J. T. (1935) J. Am. Chem. Soc. 57, 1506–1507.
[16] Bohon, R. L. and Claussen, W. F. (1951) J. Am. Chem. Soc. 73, 1571–1578.
[17] Tanford, C. (1968) Adv. Prot. Chem. 23, 121–282.