REASSOCIATION OF NUCLEIC ACIDS IN SOLUTIONS CONTAINING FORMAMIDE

H. Blüthmann, D. Brück, L. Hübner and A. Schöffski

Max-Planck-Institut für Virusforschung
Molekularbiologische Abteilung
Tübingen, Germany

Received November 14,1972

SUMMARY: The effect of formamide on the thermal stability of native and reassociated DNA-DNA duplexes and DNA-RNA hybrids has been reexamined. In contrast to McConaughy et al. (1) it was found that the Tm for native DNA of E. coli, calf and P. pallidum was reduced by 0.60°C per each 1% increase in formamide concentration. As measured by thermal stability there is no loss of specificity of the reassociation and hybridization in the formamide system under our conditions.

Formamide in nucleic acid hybridization has been introduced by Bonner et al. (2) to allow the use of lower temperatures for the reaction. The formamide system possesses great advantages over hybridization carried out at elevated temperatures. These include decreased thermal degradation of the reactants, reduced non-specific adsorption of radioactivity on membrane filters (2) and better retention of DNA on such filters (2,3).

The specificity of DNA-DNA reassociation and of DNA-RNA hybridization depends strongly on the difference between melting temperature Tm and temperature of reassociation or hybridization (4,5). To calculate the Tm in the formamide system a relationship published by McConaughy et al. (1) has been widely applied. Using this relationship Schmeckpeper et al. (6) found a disagreement between predicted and experimentally determined specificity of nucleic acid reassociation in formamide.

We have measured the thermal stability of native DNA of three different organisms between 0% and 80% formamide. Each 1% increase in formamide concentration reduces the Tm of double-stranded DNA by 0.60°C. This is in contrast

to the experiments of McConaughy et al. (1) who found a reduction of 0.72°C per 1% formamide.

By applying our relationship to the data of Schmeckpeper et al. (6) the agreement between predicted and determined specificity is much improved.

MATERIALS AND METHODS

Formamide was obtained from Merck. To remove contaminants which absorb ultraviolet light, the formamide having an optical density of 0.364 at 270 nm and of 0.230 at 275 nm was first stirred for 45 min with Ionenaustauscher V (Merck), filtered, then stirred for 20 min with charcoal and filtered again. The optical density of formamide purified by this method did not exceed 0.075 at 270 nm and 0.035 at 275 nm. The results were the same whether purified or non-purified formamide was used for the experiments.

Calf-DNA was extracted from chromatin. Lymphocyte chromatin was dissolved in 5 M urea, 0.5 M NaCl in 10^{-3} M phosphate-buffer (pH 6.8), adsorbed on hydroxylapatite, the proteins eluted first by stepwise increase of the salt and phosphate concentration, then with guanidiniumchloride and the DNA eluted batchwise with 1 M Na-phosphate (pH 6.8) (7).

E. coli-DNA and P. pallidum-DNA was extracted from whole cells with hydroxylapatite (8,9). The cells were lysed in cold buffer containing 8 M urea, 1 M $NaClO_4$, 10^{-3} M EDTA, 1% SDS and 0.2 M Na-phosphate (pH 7), mixed with 1/10 volume diethylpyrocarbonate, adsorbed on hydroxylapatite, washed first with 8 M urea, 0.2 Na-phosphate (pH 7) and then with 0.014 M Na-phosphate (pH 7). The hydroxylapatite was suspended in the last buffer, filled in a column and the DNA eluted with 0.4 M Na-phosphate (pH 7).

Calf-^{3}H-RNA complementary to calf DNA was synthesized in vitro using E. coli DNA-dependent RNA polymerase. The reaction mixture contains 0.03 Tris-HCl buffer (pH 8), 0.03 M $MgCl_2$, 0.13 M NH_4Cl, 0,5 mM ^{3}H-UTP (11.2 x 10^6

cpm/mol), 2,5 mM each of ATP, GTP, CTP; 4-5 units of enzyme per ml and 60 µg/ml of DNA. After incubation at 37°C for 2 h, the reaction was stopped by adding deoxyribonuclease I (Worthington) to a final concentration of 25 µg/ml and the incubation at 37°C continued for 30 min. The RNA was purified by gel filtration on Sephadex G-50 equilibrated with 0.05 M Na-phosphate (pH 6.8), adsorption on hydroxylapatite in 0.05 M Na-phosphate (pH 6.8) and elution with 0.20 M Na-phosphate (pH 6.8). The hydroxylapatite-column was run at 60°C. The RNA was concentrated and the buffer changed to 0.01 x SSC by gel filtration on Sephadex G-50.

P. pallidum-^{32}P-RNA has been extracted from whole cells labeled for 2 h with 3.3 mCi/ml ^{32}P in 0.01 Pipes (piperazine-N,N'-bis-(2-ethanesulfonic acid)) (10) (pH 6.5), after 7 h aeration in 0.0133 M phosphate buffer (pH 6.5) (9). The ^{32}P-labeled cells were suspended in buffer containing 0.1 M Tris, 0.005 M EDTA and acetic acid (pH 7.4), treated with diethylpyrocarbonate and lysed with 1% SDS. The RNA was extracted with phenol at 60°C. After precipitation with alcohol the RNA was purified by gel filtration on Sephadex G-50 in 0.04 x SSC, digested with carboxymethylcellulose-bound RNase to ca. 4 to 6 S and then brought to 12 mg/ml in 1 x SSC, 38% formamide.

RNA-DNA hybridization

DNA was immobilized on filters according to the method of Gillespie and Gillespie (3). DNA-RNA hybridization was carried out on 9 mm nitrocellulose filters in 100 µl 50% formamide, 2 x SSC at 41°C with calf DNA and RNA and on 25 mm filters in 600 µl 38% formamide, 1 x SSC at 49°C with P. pallidum DNA and RNA, both in duplicate and for 48 h.

Optical studies of Denaturation and Renaturation

The spectral measurements were carried out in a Gilford spectral photometer Mod. 240 equipped with a circulating water bath. Since formamide absorbs strongly at 260 nm, all optical studies described here were

monitored at 275 nm. Identical results were obtained by measuring at 270 nm.

The decrease in thermal stability of DNA as a function of formamide concentration was measured optically by denaturing DNA in various concentrations of formamide in 1 x SSC and 2 x SSC. The midpoint, Tm, of the hyperchromic transition was determined as a function of formamide concentration.

The reassociation of melted DNA was carried out in solution under the condition used for DNA-RNA hybridization for calf DNA (corresponding to Tm -20°C) and P. pallidum DNA (Tm -10°C) and for E. coli DNA in 55% formamide, 2 x SSC at 41°C (Tm -20°C) respectively in 55% formamide, 1 x SSC at 49°C (Tm -10°C). The DNA used for reassociation was sheared to ca. 3 x 10^5 MW by sonication.

Thermal Stability Measurements

The thermal stability of the reassociated DNA was determined as described above for native DNA.

To determine the thermal stability of the DNA-RNA hybrids, 6 mm pieces cut out of the incubated 25 mm filters (P. pallidum) or quarters of the 9 mm filters (calf) were heated in 50 µl of the hybridization solution lacking RNA for 30 min. The solution was then diluted with 2 ml icecold 2 x SSC, the filter pieces removed and washed with 2 x SSC and the radioactivity left on them measured.

RESULTS

Reduction of Thermal Stability by Formamide

The effect of formamide in reducing the thermal stability of double-stranded DNA was studied by determining the relationship between formamide concentration and the Tm of the DNA. The results presented in Fig. 1a and 1b show that a linear relationship exists between the Tm and the formamide concentration. The Tm of native DNA is

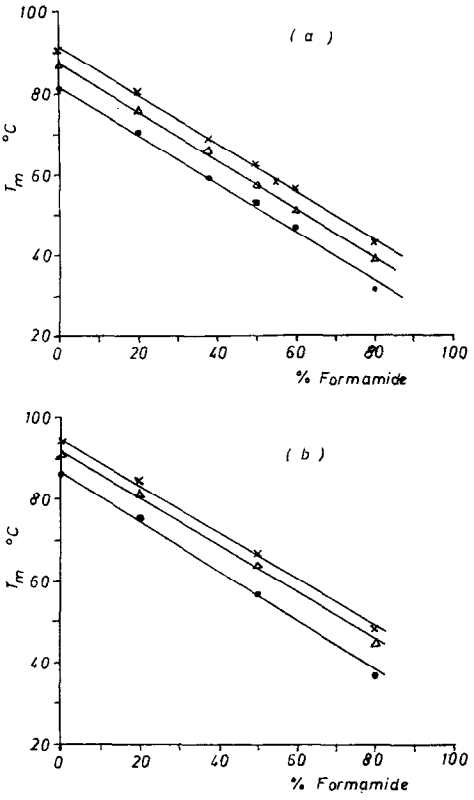

Fig. 1.

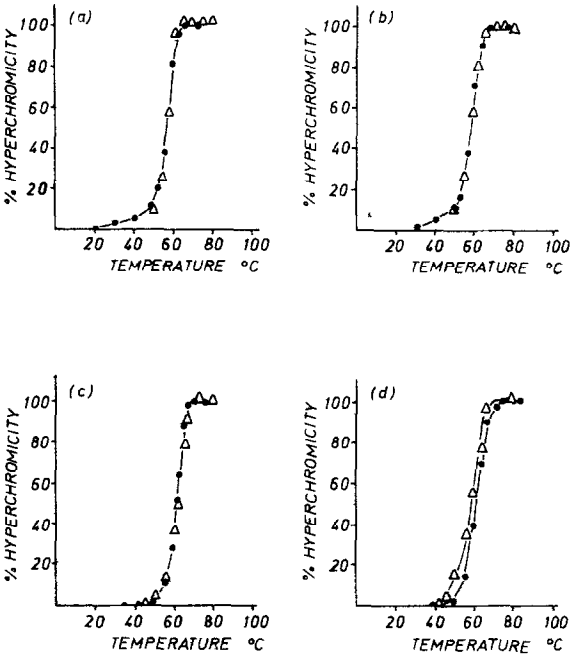

Fig. 2.

Fig. 1. Dependence of the melting temperature Tm of native DNA upon the concentration of formamide in 1 x SSC (a) and 2 x SSC (b). P. pallidum - DNA ●—●; calf - DNA Δ—Δ; E. coli - DNA x—x.

Fig. 2. Melting curves for native (●—●) and reassociated (Δ—Δ) DNA of E. coli, P. pallidum and calf.

(a) DNA from E. coli in 1 x SSC, 55% formamide at 48°C. 80% of the DNA was reassociated at Tm -10°C.

(b) DNA from P. pallidum in 1 x SSC, 38% formamide at 49°C. 40% of the DNA was reassociated at Tm -10°C.

(c) DNA from E. coli in 2 x SSC, 55% formamide at 41°C. 95% of the DNA was reassociated at Tm -20°C.

(d) DNA from calf in 2 x SSC, 50% formamide at 41°C. 50% of the DNA was reassociated at Tm -20°C.

---

reduced by 0.60 ± 0.01°C per each 1% increase in formamide concentration for E. coli-DNA, calf-DNA and P. pallidum-DNA in both 1 x SSC and 2 x SSC.

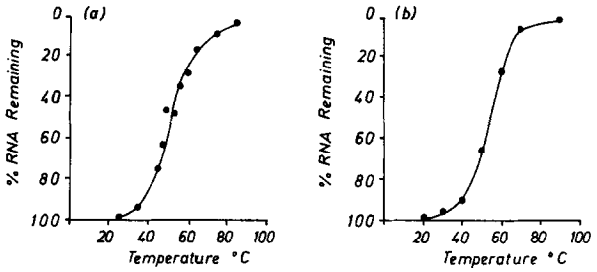

Fig. 3. Thermal stability profiles of calf DNA-RNA hybrids formed in 50% formamide, 2 x SSC at 41°C (a), and P. pallidum DNA-RNA-hybrids formed in 38% formamide, 1 x SSC at 49°C (b).

Thermal Stability of Reassociated DNA and of DNA-RNA Hybrids

The thermal stability of reassociated DNA and of DNA-RNA hybrids was determined by measuring their Tm. As can be seen from Fig. 2, there is no difference between Tm of native DNA and of DNA reassociated at Tm -10°C for E. coli and P. pallidum. Even for calf DNA reassociated at Tm -20°C the difference is 2-3°C, indicating only 3-5% mispaired bases (11).

For DNA-RNA hybrids the melting temperature Tm,i as measured on filters (Fig. 3) is higher than the incubation temperature (5°C for P. pallidum, 11°C for calf). Compared to the Tm of the corresponding DNA the Tm,i are slightly reduced (4-5°C for P. pallidum, 8-9°C for calf). It is known that the Tm,i of perfectly paired DNA-RNA hybrids is 4-5°C lower than the Tm of the corresponding native DNA (12,13). Thus for P. pallidum DNA-RNA-hybrids there are virtually no mispaired bases. The reduction in Tm,i for calf indicates only 5-7% mispaired bases (11).

REFERENCES

1. McConaughy, B.L., Laird, C.D., and McCarthy, B.J., Biochemistry 8, 3289 (1969)
2. Bonner, J., Kung, G., and Bekhor, I., Biochemistry 6, 3650 (1967)
3. Gillespie, S., and Gillespie, D., Biochem. J. 125, 481 (1971)
4. McCarthy, B.J., and McConaughy, B.J., Biochem. Genetics 2, 37 (1968)
5. Church, R.B., and McCarthy, B.J., Biochem. Genetics 2, 55 (1968)

6. Schmeckpeper, B.J., and Smith, K.D., Biochemistry 11, 1319 (1972)
7. Mrozek, S. (personal communication)
8. Britten, R.J., Pavich, M., and Smith, J., Carnegie Inst. Wash. Yearbook 68, 400 (1970)
9. Brück, D., Ph. D. Thesis, Tübingen (1972)
10. Good, N.E., Winget, G.D., Winter, W., Conolly, T.N., Izawa, S., and Singh, R.R.M., Biochemistry 5, 469 (1966)
11. Laird, C.D., McConaughy, B.L., and McCarthy, B.J., Nature 224, 149 (1969)
12. Chamberlin, M., and Berg, P., J. Mol. Biol. 8, 297 (1964)
13. Bolton, E.T., and McCarthy, B.J., J. Mol. Biol. 8, 201 (1964)