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Use of ATP, dATP and their α-thio derivatives to study DNA ligase adenylation

Alessandra MONTECUCCO, Marta LESTINGI, Guido PEDRALI-NOY, Silvio SPADARI and Giovanni CIARROCCHI*

Istituto di Genetica Biochimica ed Evoluzionistica, CNR, Pavia, Italy

Bacteriophage-T4 and human type I DNA ligases were found capable of self-adenylating upon exposure to both ribo- and deoxyribo-[α-^{35}S]thio-ATP. However, the joining reaction does not take place in the presence of the deoxyribo-triphosphates. Enzyme adenylation is reversed in all cases by an excess of PP_i, but the rate of reversion is lower with thio derivatives. Therefore thio derivatives can be used to study the adenylation of DNA ligases and to search for specific inhibitors of the first step of the ligation reaction. In addition we show that thio derivatives can be used to detect DNA ligase adenylation activity covalently bound to a solid matrix.

INTRODUCTION

The phosphodiester bond between nucleic acid chains catalysed by DNA ligase occurs through a DNA ligase–adenylate intermediate [1] and a subsequent complex involving DNA–Mg^{2+}, AMP and ligase [2–4]. Recent work in our laboratory has indicated that a number of DNA-binding drugs, including both intercalating and externally binding drugs, are capable of inhibiting the overall DNA-joining reaction catalysed by DNA ligases [5–7]. Among these drugs are the intercalators ethidium bromide, certain anthracycline derivatives and the minor groove-binding drug diamidinophenylindole. Within anthracycline derivatives, only those bearing a free amino group in the 3′-position of the sugar moiety possess an inhibitory activity towards DNA ligases [5,6]. Since the 3′-amino group of anthracyclines hangs into the minor groove of DNA during intercalation [8,9], our observations strongly supported the hypothesis that a steric occupancy of the minor groove does prevent the formation of an active DNA–enzyme complex [7].

None of the above-mentioned drugs has been found capable of inhibiting the adenylation of bacteriophage-T4 DNA ligase, the first step of DNA ligation [7]. To extend our studies to the human enzyme, usually available at lower absolute concentrations, we needed, however, a labelled cofactor with a specific radioactivity higher than that available in the tritiated form. We have therefore thought of utilizing a derivative of ATP labelled with ^{35}S in the α position [10]. Here we present evidence validating this new method for the determination of self-adenylating activities. In addition, we present new observations indicating that deoxyribo derivatives can also be used to adenylate DNA ligase activity. These methods can be used when the self-adenylating activity is either in solution or bound to a solid matrix, and proved useful in searching for, and detecting, inhibitors of DNA ligase adenylation.

METHODS

Materials

Human DNA ligase I was purified from HeLa cells [11], whereas bacteriophage-T4 DNA ligase (3000 units/mg) was purchased from Boehringer, Mannheim, Germany. ^{3}H and [α-^{35}S]thio derivatives and the unlabelled thio derivatives were purchased from Amersham International. Anthracycline derivatives were kindly supplied by Dr. Suarato, Farmitalia–Carlo Erba, Milan, Italy. Nitrocellulose membrane was obtained from Schleicher und Schüll, Dassel, Germany; all other reagents were from Merck.

DNA-joining activity

DNA-joining activities were determined by the method of Modrich & Lehman [12], according to which 1 unit of DNA ligase is the amount of enzyme activity that converts 100 nmol of poly(dA-dT) to an exonuclease III-resistant form within 30 min at 30 °C.

Adenylation of DNA ligase in solution

DNA ligases were incubated at 37 °C for 5 min in the same reaction mixture (10 μl) utilized for DNA ligation, except that cofactor and its concentration were as specified in the text. The reaction was stopped by the addition of a solution (20 μl) containing 10 mM-EDTA and 150 μg of BSA/ml; then 25 μl of the reaction mixture was spotted on to a Whatman GF/C filter; the filter was batch-washed with trichloroacetic acid, dried and counted for radioactivity.

Adenylation of DNA ligase bound to nitrocellulose filters

The nitrocellulose filter (Schleicher und Schüll) was soaked in buffer A [20 mM-Tris/HCl (pH 8.0)/50 mM-NaCl/1 mM-dithioerythritol] and mounted on a slot-blot apparatus. Samples were diluted in buffer A and applied in constant volumes (200 μl each). The membrane (10 cm^2) was then equilibrated with buffer B [50 mM-Tris/HCl (pH 7.5) 10 mM-$MgCl_2$/1 mM-dithioerythritol] for 30 min at 4 °C and then incubated overnight at room temperature with 2 ml of buffer B containing 12.5 μCi of [α-^{35}S]ATP/ml (400 Ci/mmol). The membrane was then washed for 30 min four times with a solution containing 20 mM-Tris/HCl, pH 8.0, 1 mM-dithioerythritol and 0.1 % (v/v) Tween 20, dried, and autoradiographed for 2 h at room temperature.

Reversion of enzyme adenylation with PP_i

After adenylation had occurred under the conditions described above, PP_i was added as specified in the text and incubation was continued for an additional 5 min. After this time, reactions were stopped and adenylated enzyme determined as described above.

* To whom correspondence and reprint requests should be addressed.

Table 1. Adenylation of DNA ligases with ribo and deoxyribo cofactors

Cofactor	Adenylation (pmol of cofactor/unit of enzyme)	
	Bacteriophage-T4 DNA ligase	Human DNA ligase
[^{3}H]ATP	1.17±0.11	Not detectable*
[α-^{35}S]ATP	0.7±0.09	1.8±0.08
[^{3}H]dATP	0.49±0.02	Not detectable*
[α-^{35}S]dATP	0.2±0.2	0.62±0.09

* Tritiated ribo- or deoxyribo-triphosphates do not allow significant measurement of the adenylation of the human enzyme, which is usually available at concentrations of two or three orders of magnitude lower than the bacteriophage-T4 DNA ligase.

RESULTS AND DISCUSSION

Adenylation of bacteriophage-T4 and human DNA ligases with ribo- or deoxyribo-ATP and with their corresponding α-thio derivatives

The overall DNA-joining reaction by bacteriophage-T4 and eukaryotic DNA ligases requires ATP. These enzymes are essentially inactive when dATP substitutes for ATP.

We show in Table 1 that with the bacteriophage-T4 and the human enzyme, DNA ligase–adenylate, the first covalent intermediate of the DNA-joining reaction, occurs not only when dATP replaces ATP as cofactor, but also in the presence of their corresponding ribo or deoxyribo α-thio derivatives.

The adenylation reaction is more efficient with the ribo than with the deoxyribo derivatives and less efficient with the thio derivatives.

The level of adenylation of the human enzyme increased upon dialysis of the enzyme with PP_i, a product of adenylation, suggesting that the purified enzyme is partially adenylated (Fig. 1). Adenylation of the eukaryotic enzyme is linear with the time of incubation at 37 °C up to 30 min, a kinetic feature that distinguishes the human ligase from the bacteriophage-T4 enzyme, which shows no detectable kinetics, reaching the maximal level of adenylation at 0 °C at zero time.

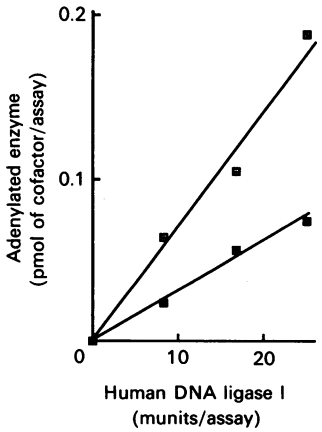

Fig. 1. Adenylation of human DNA ligase I upon exposure to [α-^{35}S]ATP

Incubation was performed with the enzyme dialysed at −20 °C against 10 mM-sodium phosphate (pH 7.2)/150 mM-NaCl/50% (v/v) ethylene glycol (■) or against the same buffer containing 50 μM-sodium pyrophosphate (⊡).

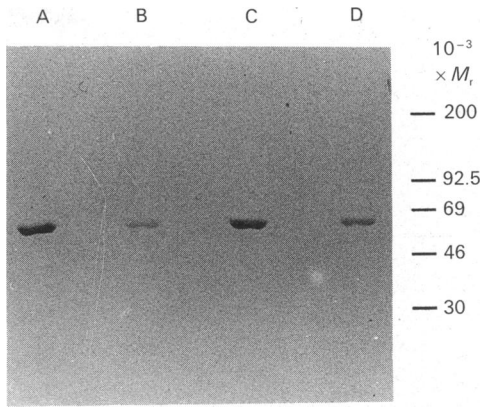

Fig. 2. Denaturing PAGE of adenylated bacteriophage-T4 DNA ligase

The bacteriophage enzyme was incubated with either [α-^{35}S]thio-ATP (lanes A and B) or [α-^{35}S]thio-dATP (lanes C and D) and then with PP_i (lanes B and D) as described in the Methods section. Samples were then subjected to SDS/PAGE. Marker proteins run in adjacent lanes were myosin, phosphorylase *b*, albumin, ovalbumin and carbonic anhydrase (in descending order of M_r).

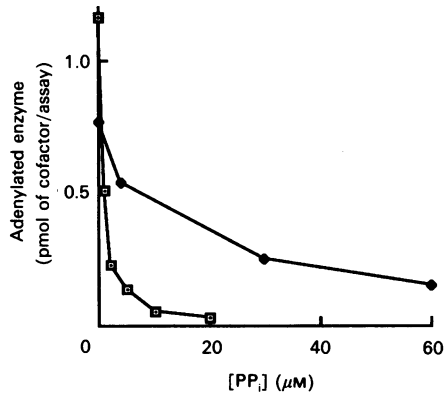

Fig. 3. Reversion of adenylation of bacteriophage-T4 DNA ligase

Incubation was performed with either [^{3}H]ATP (⊡) or [α-^{35}S]thio-ATP (◆) and then with increasing concentrations of PP_i. Samples were then processed as described in the Methods section.

Bacteriophage-T4 DNA ligase adenylated with both ribo- and deoxyribo- [α-^{35}S]thio-AMP appears as a single autoradiographic band after SDS/PAGE analysis (Fig. 2, lane A). The occurrence of monophosphate exchange by PP_i is evidenced by the almost complete disappearance of such a band (Fig. 2, lane B). However, the displacement from the adenylated enzyme by PP_i is less efficient in the case of the thio-dAMP, since after gel analysis the resulting reduced autoradiographic band is more evident than the one obtained with the thio-AMP (Fig. 2, lanes C and D). In turn, the displacement of thio-AMP from the adenylated enzyme is less efficient than that observed with AMP, as shown in Fig. 3. The gel analysis also indicates that the binding of dAMP to the enzyme is covalent.

[α-^{35}S]Thio-ATP, but not dATP or [α-^{35}S]thio-dATP, can substitute for ATP in driving the overall joining reaction

The thio derivative of ATP, but not dATP or its thio derivative, was also found capable of driving the entire reaction of ligation, although at a rate lower than that obtained in the presence of ATP (Fig. 4). Our observation that adenylation of DNA ligase occurs in the presence of dATP or thio dATP, coupled with the

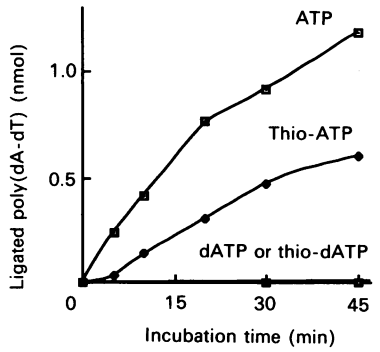

Fig. 4. Kinetics of poly(dA-dT) circularization catalysed by bacteriophage-T4 DNA ligase in the presence of various cofactors

The cofactor concentration was 1 mM in all cases.

fact that the chemically generated DNA–deoxyadenylate (DNA–dAMP) sustains the phosphodiester-bond formation with release of dAMP [13], suggests that dATP exerts its major inhibitory effect upon the second step of DNA ligation, that is, the formation of the complex involving DNA–Mg^{2+}, AMP and ligase, perhaps by stabilizing the dAMP–enzyme intermediate and preventing the adenylyl transfer from ligase to DNA.

The above reactions of adenylation with thio derivatives are quickly reversed when an excess of PP_i is added to the reaction mixture (Figs. 2 and 3).

DNA ligase retains its self-adenylating activity when covalently bound to a solid matrix

The α-^{35}S-labelled derivatives have been found useful in detecting self-adenylating activities after their binding to a solid matrix. In the case shown in Fig. 5, three increasing amounts of bacteriophage-T4 DNA ligase (1.6, 6.4 and 11.2 μg) were bound to a nitrocellulose membrane by means of the slot-blot technique. The membrane was then incubated in the presence of [α-^{35}S]thio-ATP. Autoradiography of the membrane revealed that the enzyme is still active after adhesion to the solid matrix and that the amount of adenylated product is proportional to the amount of protein linked to the matrix.

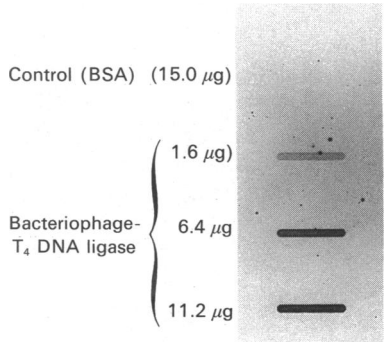

Fig. 5. Revelation of self-adenylating activity on the nitrocellulose filter

Aliquots of proteins were bound to a nitrocellulose filter through a slot-blot device. Incubation was performed in the presence of [α-^{35}S]thio-ATP as described in the Methods section and then autoradiographed.

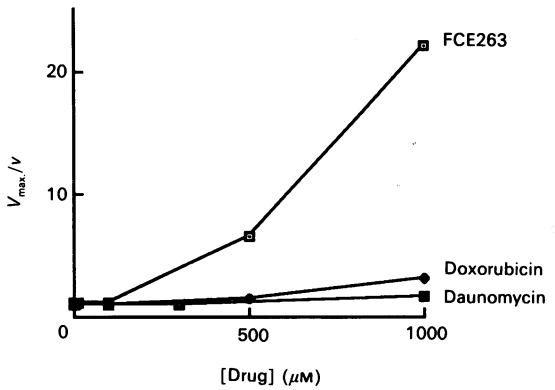

Fig. 6. Inhibition of human DNA ligase I adenylation by anthracycline derivatives

Enzyme adenylation was monitored as trichloroacetic acid-precipitable radioactivity after incubation of the human enzyme with [α-^{35}S]thio-ATP for 5 min at 37 °C.

Use of thio-ATP in the search for inhibitors of DNA ligase adenylation

The possibility of generating a [^{35}S]thio-adenylate–DNA ligase intermediate favours its use in the search for specific inhibitors of the adenylation step, especially in the case of the human DNA ligase, which, in contrast with the bacteriophage-T4 DNA ligase, clearly shows kinetics of adenylation, as mentioned above. In this regard we have utilized [α-^{35}S]thio-ATP as labelled cofactor to analyse the effect on the DNA ligase adenylation of several anthracycline derivatives that we have recently reported as inhibiting the overall DNA-joining reaction [5,6]. An example of such analysis is presented in Fig. 6. Compound FCE263, 3′-deamino-4′-deoxy-4′-aminodoxorubicin, when tested in an adenylation assay for human DNA ligase, shows a much stronger inhibitory action than its parental derivative doxorubicin or the related compound daunomycin. This effect is even more noteworthy if one considers that compound FCE263 is the least potent of the three in inhibiting the overall reaction of DNA joining (results not shown). This technique will be useful for detecting more specific inhibitors of DNA ligases.

During the preparation of this manuscript we were informed by Dr. J. M. Rossignol, Laboratoire de Biologie Moléculaire de la Réplication, CNRS, Villejuif, France, that, in his laboratory, α- and γ-thio-ATP have recently been used to study the adenylation of rat DNA ligase and that a paper on this topic has been submitted for publication.

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REFERENCES

1. Lehman, I. R. (1974) Science **186**, 790–797
2. Teraoka, H., Sawai, M. & Tsukada, K. (1983) Biochim. Biophys. Acta **747**, 117–122
3. Montecucco, A. & Ciarrocchi, G. (1988) Nucleic Acids Res. **16**, 7369–7381
4. Montecucco, A., Pedrali-Noy, G., Spadari, S. & Ciarrocchi, G. (1988) Biochim. Biophys. Acta **951**, 330–334
5. Montecucco, A., Pedrali-Noy, G., Spadari, S., Zanolin, E. & Ciarrocchi, G. (1988) Nucleic Acids Res. **16**, 3907–3918
6. Montecucco, A., Pedrali-Noy, G., Spadari, S., Zanolin, E., Chiesa, M. T. & Ciarrocchi, G. (1989) J. Chemother. **4**, 1138–1139

7. Montecucco, A., Pedrali-Noy, G., Spadari, S., Lestingi, M. & Ciarrocchi, G. (1990) Biochem. J. **266**, 379–384
8. Wang, A. H.-J. (1987) Nucleic Acids Mol. Biol. **1**, 53–69
9. Moore, M. H., Hunter, W. N., Langlois d'Estaintot, B. & Kennard, O. (1989) J. Mol. Biol. **206**, 693–705
10. Eckstein, F. & Goody, R. S. (1976) Biochemistry **15**, 1685–1691
11. Spadari, S., Ciarrocchi, G. & Falaschi, A. (1971) Eur. J. Biochem. **22**, 75–78
12. Modrich, P. & Lehman, I. R. (1970) J. Biol. Chem. **245**, 3626–3634
13. Harvey, C. L., Gabriel, T. F., Wilt, E. M. & Richardson, C. C. (1971) J. Biol. Chem. **246**, 4523–4530

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