THE FORMATION OF A LONG-LIVED COMPLEX BETWEEN AN ATP-DEPENDENT DEOXYRIBONUCLEASE AND DNA

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1. Introduction

ATP-dependent deoxyribonucleases, all apparently with essentially the same properties, have been reported from several genera of bacteria [1–8]. In the case of two of these organisms, *Escherichia coli* and *Diplococcus peneumoniae*, evidence for the involvement of this enzyme in recombination and repair of DNA has been obtained [5, 6, 9–11]. Investigations into the action of the enzyme from *Mycobacterium smegmatis* have shown that it has a marked preference for double-stranded DNA, that it degrades from the ends of strands, that it attacks both strands more-or-less simultaneously, and that it releases a mixture of short-chain oligonucleotides [12–14]. Similar findings have been obtained for the enzyme from *Micrococcus lysodeikticus* [7, 8].

We report here the results of some investigations with the *M. smegmatis* enzyme, designed to determine whether a complex between it and the DNA could be demonstrated and whether ATP plays a role in the formation of such a complex.

2. Materials and methods

The purification of the enzyme started with extracts of iron-limited *M. smegmatis*, which contain about 5-fold higher activity than normal extracts of this organism [12]. The enzyme was purified 180-fold further by streptomycin treatment, protamine precipitation, hydroxyapatite treatment, fractionation on DEAE-cellulose and chromatography on Sephadex G-200, to give a specific activity of about 20,000 units/mg of protein. The procedure was a modification of that of Winder and Lavin [13] and will be described in detail elsewhere. The enzyme was assayed as described previously [13], and one unit of enzyme is defined as that amount which released 1 nmole of acid-soluble deoxyribose from DNA in 30 min under the defined conditions. Other experimental details are given in the appropriate tables.

^{32}P-DNA was prepared from *E. coli* by the method of Marmur [15]. The unlabelled DNA employed was highly-polymerized DNA from fish soft roe (Type VI from Sigma Chemical Company).

3. Results

Experiments of the type illustrated in table 1 showed that, when a small amount of ^{32}P-DNA was incubated for a brief period with the deoxyribonuclease in the presence of ATP and Mg^{2+}, a much increased proportion of the DNA became adsorbable to nitrocellulose filters. That this was due to formation of a complex between DNA and enzyme, and not due to alteration in the DNA, was shown by deproteinizing other samples by treatment with phenol-SDS, pH 9.0, dialyzing, and passing through membrane filters: the DNA so purified was not adsorbed to the filters. That the binding was not due to the formation of a non-specific complex with protein was shown by the use of serum albumin in place of enzyme (table 1). The results in table 1 also showed that little binding occurred when ATP was omitted, but that about half the binding occurred in the absence

Table 1
Binding of ^{32}P-DNA to nitrocellulose filters after exposure to ATP-dependent deoxyribonuclease.

Reaction mixture	DNA retained on filter (nmoles)	DNA retained, control subtracted (nmoles)
DNA + ATP + Mg^{2+} (control)	0.176	–
Bovine serum albumin + DNA + ATP + Mg^{2+}	0.190	0.014
Enzyme + DNA	0.260	0.084
Enzyme + DNA + Mg^{2+}	0.366	0.190
Enzyme + DNA + ATP	0.663	0.487
Enzyme + DNA + ATP + Mg^{2+}	0.905	0.729

The complete reaction mixture contained in 1 ml: 2 μmole of ATP, 20 μmole of $MgCl_2$, 75 μmole of Tris-HCl buffer (pH 8.0), 50 μmole of KCl, 0.15 μmole of dithiothreitol, 1.89 nmole of ^{32}P-DNA (1360 cpm) from *E. coli*, and 298 units of enzyme. When bovine serum albumin replaced the enzyme, 50 μg was used, The mixture was incubated at 30° for 30 sec, chilled rapidly and passed at about 1 ml per min through a Millipore filter, type HAWP, 25 mm in diameter, which had been pre-washed with 10 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 0.26 mM EDTA and 1 ml dithiothreitol. After the sample, the filter was washed with a further 10 ml of the same buffer. The filters were dried and the ^{32}P counted in a toluene-based scintillation fluid. Assays were carried out in triplicate.

Table 2
Binding of ATP-dependent deoxyribonuclease to nitrocellulose filters in the presence of DNA.

Reaction mixture	Enzyme retained on filter (units)
Enzyme + ATP + Mg^{2+}	0.0
Enzyme + DNA	0.7
Enzyme + DNA + Mg^{2+}	0.7
Enzyme + DNA + ATP	6.9
Enzyme + DNA + ATP + Mg^{2+}	10.3

The complete reaction mixture (298 units of enzyme), incubation and filtration were as described in the legend to table 1. These were done in triplicate. The filters were then cut up and the enzyme attached to them was assayed by placing them in assay mixtures which contained in 1 ml: 2 μmole of ATP, 20 μmole of $MgCl_2$, 75 μmole of Tris-HCl buffer (pH 8.0), 0.15 μmole of dithiothreitol, 18.9 nmole of ^{32}P-DNA (13,600 cpm) and 50 μg of bovine serum albumin. The mixtures were incubated at 37° for 30 min, the reaction was stopped by the addition of 0.5 ml of 1 M $HClO_4$ and, after centrifugation, 0.5 ml samples were taken for counting in Bray's fluid. The results were calculated from a standard curve prepared by incubating various amounts of enzyme in the same assay mixture.

of Mg^{2+}. It is quite likely that the small amount of binding which occurred in the absence of ATP was due to contamination of the preparation with other proteins which interact with DNA.

Thus, ATP probably plays an essential role in the formation of the enzyme–DNA complex, whereas Mg^{2+} can be replaced to a substantial extent by other ions or is involved only in the conversion of the complex from a less stable to a more stable form.

The formation of a DNA–enzyme complex was also shown by the fact that enzyme activity could be recovered from the filters through which the enzyme–DNA–ATP–Mg^{2+} had been passed (table 2). The results on recovery of enzyme from the filters, like those on recovery of DNA, showed a strong requirement for ATP for complex formation, and less evidence of a requirement for Mg^{2+}. A much smaller proportion of the enzyme than of the DNA present in the reaction mixture appeared to be bound to the filters, suggesting that the enzyme molecules were present in excess over enzyme-binding sites in the DNA present in the incubation mixture.

The results in table 3 show that the enzyme present in the filter-bound complex could break down the DNA present in the complex, when the filters were transferred to a fresh ATP–Mg^{2+}-containing buffer mixture, but not to an appreciable extent when ATP was omitted. Although the percentage of the bound DNA which was rendered acid-soluble by the bound enzyme may not seem large, the bound enzyme was in fact between one-third and one-half as active with the bound DNA as with DNA added as described in table 2, in spite of the fact that 20 to 30 times more DNA was available in the latter case. This suggested that the enzyme attacked the DNA in the complex particularly efficiently, and hence did not detach from the DNA between each hydrolytic step.

In order to test this last conclusion, experiments of the type illustrated in table 4 were carried out. The idea behind these experiments was to compare the effect on the breakdown of ^{32}P-DNA of an excess of unlabelled DNA, added either before the enzyme or after a brief period had been allowed for

Table 3
Release of acid-soluble ^{32}P from filter-bound DNA by filter-bound enzyme.

Reaction mixture used for binding	Percentage of bound DNA rendered acid-soluble on subsequent incubation	
	In absence of ATP	In presence of ATP
Enzyme + DNA	1.9	6.5
Enzyme + DNA + Mg^{2+}	4.1	9.8
Enzyme + DNA + ATP	1.0	14.0
Enzyme + DNA + ATP + Mg^{2+}	1.3	14.3

The complete reaction mixture (298 units of enzyme and 1.89 nmole of ^{32}P-DNA), incubation and filtration were as described in the legend to table 1. These were done in triplicate. The filters were then cut up and placed in assay mixtures as in table 2, except that they contained no DNA and that ATP was omitted from half of the mixtures. These were incubated and the acid-soluble ^{32}P counted as in table 2. The acid-soluble ^{32}P was calculated as a percentage of that bound to the filters, after correction for different counting efficiencies in the two systems.

Table 4
Non-equilibration between enzyme-bound ^{32}P-DNA and unlabelled DNA subsequently added.

Addition of unlabelled DNA to mixture	Period of reaction (min)	^{32}P-DNA solubilized (nmole)
None	0.5	0.99
None	10	8.85
Unlabelled DNA added before enzyme	10	1.03
Unlabelled DNA added after 30 sec reaction	10	3.71

The reaction mixture contained in 1 ml: 2 μmole of ATP, 20 μmole of $MgCl_2$, 75 μmole of Tris-HCl buffer (pH 8.0), 50 μmole of KCl, 0.15 μmole of dithiothreitol, 38 nmole (8340 cpm) of ^{32}P-DNA and 426 units of enzyme. When unlabelled DNA was also added, at time zero or after 30 sec, 1 μmole was used. Mixtures were incubated at 37°. The reaction was stopped, at time zero, 30 sec or 10 min, by addition of 0.5 ml of 1 M $HClO_4$ and, after centrifugation, samples were taken for counting in Bray's fluid. The counts obtained were corrected for quenching, converted to nmoles of ^{32}P-DNA, and zero times values were subtracted.

the formation of a complex between the enzyme and the ^{32}P-DNA. The results showed that, as would be expected, when the ^{32}P-DNA was diluted with unlabelled DNA before the introduction of the enzyme, the extent of breakdown of the ^{32}P-DNA was substantially reduced. However, when the unlabelled DNA was added after the enzyme and the ^{32}P-DNA had been allowed to interact for a brief period, its effect was less marked, even when allowance was made for the breakdown of ^{32}P-DNA which had already taken place. This demonstrated that the enzyme, once it had attached to a particular molecule of DNA, remained attached to it until at least an appreciable proportion of it had been degraded.

However, the addition of unlabelled DNA after the enzyme did have an effect (table 4). Further experimentation will be required to determine whether this was due to the enzyme's having a certain tendency to drop off, or to transfer directly to another DNA molecule, from the DNA to which it was attached, or whether it was due to enzyme molecules having completed the degradation of their molecules of DNA during the incubation period. It should be possible to settle this by examination of the kinetics of breakdown of labelled DNA after addition of unlabelled DNA.

4. Discussion

The results described above show that a complex is formed between this enzyme and DNA. Consequently, the approaches described in this communication provide a means of separately studying the formation and breakdown of this complex, and should be useful in the further elucidation of these processes.

The results show that one of the roles of ATP in the action of this enzyme is in the formation of the enzyme–DNA complex, though, in view of the fact that further ATP is required for the conversion of enzyme-bound DNA to acid-soluble fragments (table 3) and since it is already known that there is about a 3:1 ratio between molecules of ATP used and phosphodiester bonds in the DNA split [13, 14], this is clearly not the only role of ATP.

The data in tables 1 and 2, combined with a determination of the degree of purity of the enzyme, its molecular weight and the molecular weight of the DNA employed, would permit the calculation of the number of enzyme molecules bound per molecule of DNA. We do not yet have sufficiently accurate data on all these points.

An interesting conclusion from the experiments described above is that the complex between a molecule of enzyme and a molecule of DNA continues in existence until the molecule of DNA is substantially, and perhaps entirely, degraded. This knowledge should be important in interpreting the kinetics of action of this enzyme and may also have an important bearing on the biological role of the enzyme.

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