STUDIES ON THE AFFINITY OF CHICK EMBRYO DNA LIGASE FOR RIBONUCLEOTIDES AND DEOXYRIBONUCLEOTIDES

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1. Introduction

We have recently described a five-step purification and the properties of a soluble chick embryo DNA ligase (EC 6.5.1.1) [1]. This enzyme, like the other previously described enzymes from T_4 and T_7 infected *Escherichia coli* and all the eukaryotic DNA ligases [2–8], has been shown to use ATP as cofactor [1]. Moreover, chick embryo DNA ligase was inhibited, with low K_i (4×10^{-6} M), by dATP and this observation has made possible a single step purification of the enzyme [9]. The mechanism of this inhibition and binding is, however, still unclear and we decided to study it using equilibrium dialysis.

2. Materials and methods

Electrophoretically pure DNA ligase was obtained as already described, mol. wt 165 000 [1]. Radioactive ribonucleotides, [2-^{3}H]adenosine monophosphate (18 Ci mmol^{-1}), [2-^{3}H]adenosine diphosphate (15 Ci mmol^{-1}), and [2-^{3}H]adenosine (25 Ci mmol^{-1}), were obtained from Amersham Radiochemical Centre (Amersham, England). Radioactive deoxyribonucleotides, [8-^{3}H]deoxyadenosine monophosphate (4.6 mCi mmol^{-1}) and deoxyadenosine diphosphate (5 mCi mmol^{-1}) were from Amersham Radiochemical Centre. [^{3}H-G]Deoxyadenosine (20 Ci mmol^{-1}), [2,8-^{3}H]adenylylimidodiphosphate (α,β) (7.5 mCi mmol^{-1}) were from New England Nuclear Corporation. [2,8-^{3}H]Deoxyadenylylimidodiphosphate (α,β) was a gift from New England Nuclear Corporation and had a specific activity of 1.1 mCi mmol^{-1}. The corresponding non-radioactive compounds were all purchased from Sigma with the exception of the ATP analogs and dATP analogs, which were gifts from Dr Forterre (IRBM, Paris).

In the equilibrium dialysis experiments, the enzyme in 50 mM Tris–HCl buffer, pH 7.6, was dialysed against the ribonucleotides or deoxyribonucleotides at various concentrations in the same buffer. The method used followed that described by Koshland et al. [10]. At the start of dialysis, the first compartment (1 ml) contained 10^{-6} M to 5×10^{-5} M non radioactive nucleotides plus 1 nmol corresponding radioactive compound. The second compartment (1 ml) contained the enzyme at a concentration equivalent to that of the nucleotide in the first compartment.

To establish that equilibrium had been achieved in 24 h at 4°C a control was run with Tris–HCl buffer alone instead of the enzyme solution. After 24 h the radioactivity in both compartments was found to be equal, within the experimental error (5%).

The radioactivity was counted on 100 μl aliquots from each compartment. The solutions were spotted onto Millipore filters, completely desiccated and the radioactivity measured in a liquid scintillation counter.

Thin-layer chromatography of the dialyzed material showed that both in the presence and in the absence of enzyme there was no significant degradation of nucleotides and/or ATP or dATP analogs.

3. Results and discussion

A typical Scatchard plot is shown in fig.1. The

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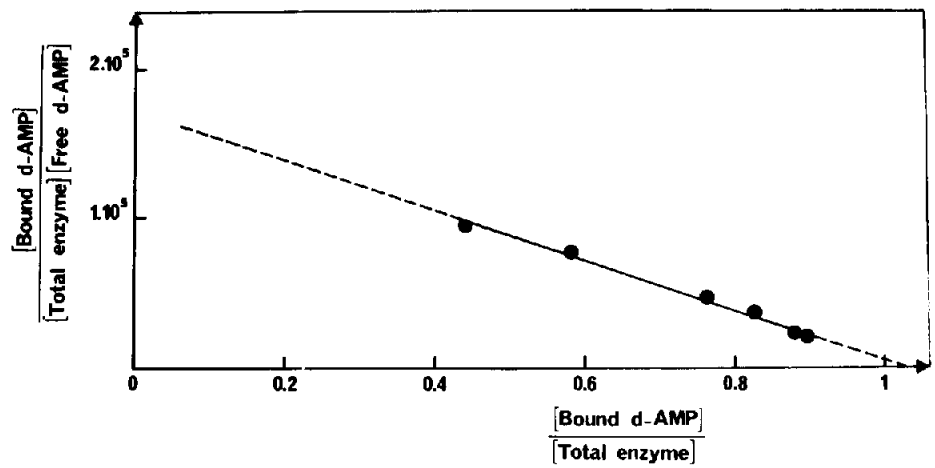

Fig.1. Scatchard plot of equilibrium dialysis data at 4°C for [^{3}H]deoxyadenosine monophosphate–DNA ligase system. The medium contained 50 mM Tris–HCl buffer, pH 7.6; 5 × 10^{-6} M DNA ligase, 10^{-6} M to 5 × 10^{-5} deoxyadenosine monophosphate. Represented in ordinate is the ratio of bound dAMP to total enzyme multiplied by free dAMP. Represented in abcissis is the ratio of bound dAMP to total enzyme concentration.

intercept on the abscissa gives the number of binding sites.

In this experiment the enzyme concentration was 5 × 10^{-6} M and the intercept corresponds to one binding site per enzyme molecule. From the slope of the Scatchard plot, the dissociation constant of the DNA ligase–dAMP complex was calculated to be 7 × 10^{-6} M. Similar experiments were done with other ribonucleotides, deoxyribonucleotides and analogs. The results are shown in table 1. For all the compounds studied only one binding site was found per enzyme molecule. For the ribonucleotides the K_d values ranges from 9 × 10^{-5} M to 4 × 10^{-5} M as the number of phosphate groups in the ligand increased. For the deoxyribonucleotides the K_d values were from 8 × 10^{-6} M to 4.7 × 10^{-6} M as the number of phosphates increased. The corresponding $\Delta G'_0$ values do not differ greatly with increasing numbers of phosphate residues.

To elucidate the role of the phosphate moiety in the binding equilibrium dialysis was also done in the presence of pyrophosphate. As shown in fig.2, there was no change in the apparent number of binding sites in the presence of 10^{-5} M, 10^{-4} M or 10^{-3} M sodium pyrophosphate. Only a slight increase in the K_d value (from 8 × 10^{-6} M to 1.2 × 10^{-5} M for

Table 1
Dissociation constants and $\Delta G'_0$ values of DNA ligase for nucleotides and deoxynucleotides

Nucleotides	K_d	$\Delta G'_0$ cal mol^{-1}	Deoxynucleotides	K_d	$\Delta G'_0$ cal.mol^{-1}
Adenosine	9 × 10^{-5} M	14 636	d-Adenosine	0.8 × 10^{-5} M	6755
Adenosine monophosphate	7.5 × 10^{-5} M	14 036	d-Adenosine monophosphate	0.7 × 10^{-5} M	6305
Adenosine diphosphate	5 × 10^{-5} M	12 685	d-Adenosine diphosphate	0.6 × 10^{-5} M	5779
Adenylyl α,β imidodiphosphate	4 × 10^{-5} M	12 009	d-Adenylyl α,β imidodiphosphate	0.47 × 10^{-5} M	5253

Values are for 4°C

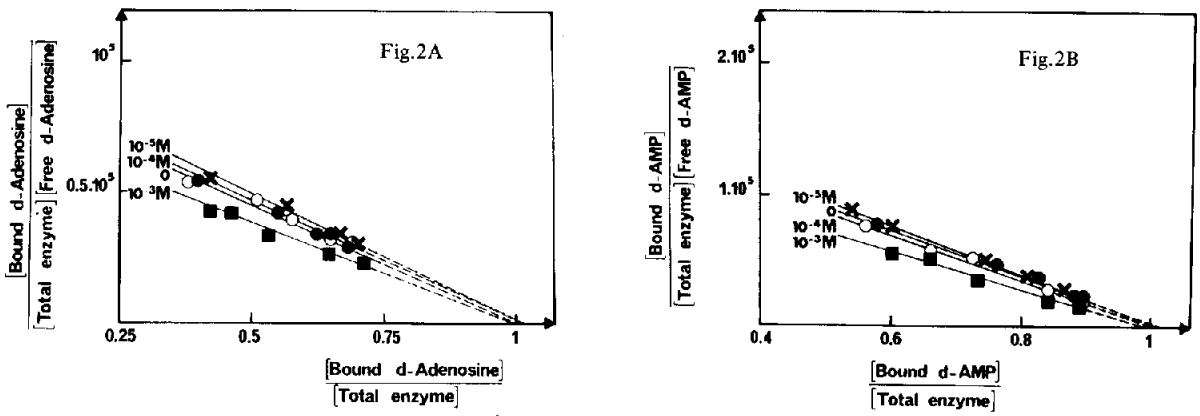

Fig.2A. Scatchard plot of equilibrium dialysis data at 4°C for [^{3}H]deoxyadenosine–DNA ligase system. The medium contained 50 mM Tris–HCl buffer pH 7.6; 10^{-5} M DNA ligase and 10^{-6} M to 5×10^{-5} M deoxyadenosine. Represented in ordinate is the ratio of bound d-adenosine to total enzyme multiplied by free d-adenosine. Represented in abcissis is the ratio of bound d-adenosine to total enzyme concentration. This experiment in the presence of 10^{-5} M, 10^{-4} M and 10^{-3} M pyrophosphate. Fig.2B. Scatchard plot of equilibrium dialysis data at 4°C for [^{3}H]deoxyadenosine monophosphate–DNA ligase system. The medium contained 50 mM Tris–HCl buffer, pH 7.6; 5×10^{-6} M DNA ligase and 10^{-6} M to 5×10^{-5} M deoxyadenosine monophosphate. Represented in ordinate is the ratio of bound dAMP to total enzyme multiplied by free dAMP. Represented in abcissis is the ratio of bound dAMP to total enzyme concentration. This experiment in the presence of 10^{-5} M, 10^{-4} M and 10^{-3} M pyrophosphate.

deoxyadenosine and 7×10^{-6} M to 10^{-5} M for dAMP) was observed at 10^{-3} M pyrophosphate. This result is in good agreement with the observed inhibition of chick embryo DNA ligase activity (30% decrease) in the presence of 10^{-3} M sodium pyrophosphate [1]. Similar results were obtained with dAMP in the presence of sodium pyrophosphate. These results are consistent with the hypothesis that the ligase binds to the adenosine moiety. This is similar to the conclusions from the observations made with phage T_4 ligase by Harvey et al. [11]. Further, the existence of only one binding site and the very small increase in affinity of the enzyme for the mono-, di- and triphosphonucleotides indicate that the enzyme reacts with the nucleoside part of the molecule.

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