Simple and fast purification of *Escherichia coli* adenylate kinase

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Received 26 January 1983

Adenylate kinase from *E.coli* (strains CR341 and CR341 T28, a temperature-sensitive mutant) was purified by a two-step chromatographic procedure. The enzyme from crude extracts of both mutant and parent strain was bound to blue–Sepharose at pH 7.5, thereafter specifically eluted with 0.05 mM P^1,P^5-di(adenosine-5′)pentaphosphate. A second chromatography on Sephadex G-100 yielded pure enzyme. *E.coli* adenylate kinase was strongly inhibited by P^1,P^5-di(adenosine-5′)pentaphosphate (K_i 0.6 μM for adenylate kinase of strain CR341 and 2.1 μM in the case of mutant enzyme). After denaturation in 6 M guanidinium hydrochloride both mutant and parent adenylate kinase returned rapidly to the native, active state by dilution of guanidinium hydrochloride.

Adenylate kinase Escherichia coli *Two-step purification* *blue–Sepharose* P^1,P^5-*di(adenosine-5′)pentaphosphate*

1. INTRODUCTION

Adenylate kinase (EC 2.7.4.3) is an essential enzyme involved in the interconversion of adenine nucleotides in energetically active tissues [1]. The purification of adenylate kinase from different sources generally involves acid treatment of the crude extract, selective precipitation by ammonium sulfate, ion-exchange chromatography and gel filtration [1–6]. One important improvement in the purification of adenylate kinase was its ability to bind to blue–Sepharose [7–9], followed by elution with natural nucleotides, with or without the addition of salt. The enzyme from *Escherichia coli* represents ~0.1–0.2% of the soluble protein. However, the reported yields are quite low (~5%) [10–12]. Hence, physico-chemical studies of bacterial adenylate kinase have been limited.

Abbreviations: blue–Sepharose, Cibacron blue 3G-A–Sepharose CL-6B; Ap_5A, P^1,P^5-di(adenosine-5′)-pentaphosphate; GdmCl, guanidinium hydrochloride; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); SDS, sodium dodecylsulfate

Here, we report a simple and fast two-step procedure for purification of *E. coli* adenylate kinase, suitable for both small- and large-scale purposes.

2. MATERIALS AND METHODS

2.1. *Chemicals*

All commercial nucleotides, substrates and coupling enzymes were products of Boehringer (Mannheim). DTNB came from Sigma (St Louis MO). Blue–Sepharose was obtained from Pharmacia (Uppsala). GdmCl (fluorimetrically pure) was from Carlo Erba (Milan).

2.2. *Growth of bacteria and preparation of cell-free extracts*

E. coli K12 strains used in this work were CR341 and CR342 T28 (the temperature-sensitive mutant), both F^- *thr leu* B_1 *thy lacY met* [13]. Cells were grown aerobically at 37°C (strain CR341) or 32°C (strain CR341 T28) in LB medium [14] supplemented with 0.1 mg thymine/ml, until late logarithmic phase of growth. Bacteria from 2 l culture were harvested by centrifugation at 5000 × *g* for 30 min, washed twice with 50 mM Tris–HCl

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

(pH 7.4), and disrupted by sonication in the same buffer at 20000 Hz and 100 W (6 pulses of 15 s each). Cell debris was removed by centrifugation at 13500 × *g* for 20 min. The extract could be stored for at least two weeks at −30°C without any loss of activity.

2.3. *Analytical procedures*

Adenylate kinase activity was measured in both directions using a coupled colorimetric assay [15] in the direction of ADP formation, or a spectrophotometric assay in the direction of AMP and ATP formation. In the first case the reaction mixture contained in 1 ml final vol. 50 mM Tris–HCl (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 1 mM ATP, 1 mM AMP and 1 unit of pyruvate kinase. The reaction was triggered by the addition of pure or crude adenylate kinase preparation. After 2–10 min incubation at 27°C, 0.1 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl was added. After another 15 min incubation at 27°C, 0.5 ml 2.5 N NaOH in 50 mM EDTA were added and the absorbance read at 460 nm. In the second case the reaction medium contained in 1 ml final vol., 50 mM Tris–HCl (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 10 mM glucose, 0.4 mM $NADP^+$, 2 mM ADP and 3.6 units each of hexokinase and glucose-6-phosphate dehydrogenase. The reaction was triggered by the addition of pure or crude adenylate kinase preparation. Absorption increase at 340 nm and 27°C was followed with a Gilford 240 spectrophotometer equipped with a recorder (full scale deflection 0.2 absorbance units). Proteins were measured as in [16]. SDS–Polyacrylamide slab gel electrophoresis was run for 3.5 h on a gel containing 10% acrylamide, 0.25% bisacrylamide and 0.1% SDS.

3. RESULTS

3.1. *Purification of* E. coli *adenylate kinase*

The bacterial extract (~25 ml containing 16–20 mg of protein/ml) was loaded onto a blue–Sepharose column of 1.2 × 9 cm, equilibrated with 50 mM Tris–HCl (pH 7.4) at 10 ml/h. The column was washed with 30 ml of the same buffer until no protein could be detected in the eluate. Under these conditions, > 95% of the adenylate kinase binds to blue–Sepharose, while 70–80% of the proteins in the extract appear in the pass-through fraction. Adenylate kinase was eluted with 20 ml 0.05 mM Ap_5A in 50 mM Tris–HCl (pH 7.4) at 10 ml/h. Between 70–80% of the adenylate kinase was eluted in total vol. 6–8 ml. The enzyme was directly applied to a 2 × 40 cm Sephadex G-100 column equilibrated with 50 mM Tris–HCl (pH 7.4) and fractions of 1.7 ml were collected at 10 ml/h. The peak fractions containing adenylate kinase (6.8–7.5 ml) were pooled, precipitated by dialysis against saturated ammonium sulfate, and stored at 4°C.

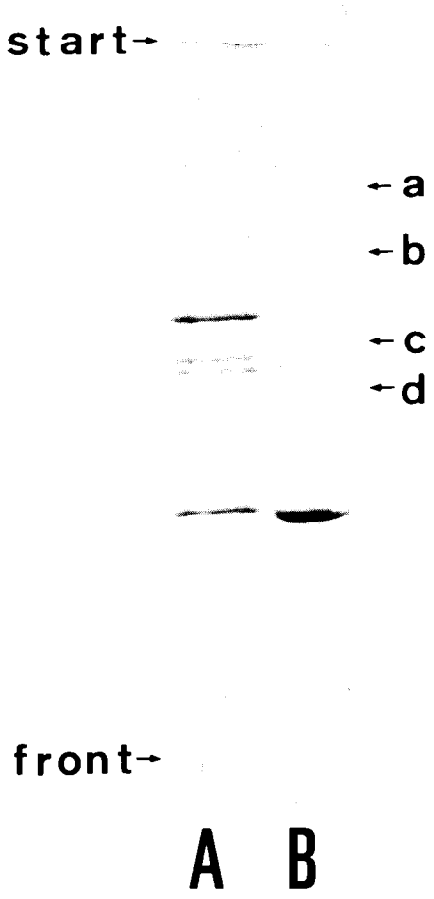

Fig.1. SDS–polyacrylamide gel electrophoresis of fractions obtained during the purification of adenylate kinase from *E. coli* (strain CR341). The arrows indicate the standard proteins, from top to bottom: (a) phosphorylase *a* (94000); (b) bovine serum albumin (68000); (c) fumarase (48500); (d) aldolase (40000). (A) Blue–Sepharose chromatography (8 μg protein); (B) Sephadex G-100 chromatography (8.8 μg protein).

In a typical experiment, starting from 450 mg protein from *E. coli* strain CR341 T28 (spec. act. 0.15 units/mg protein, in the direction of ATP formation), we obtained 0.6 mg pure adenylate kinase having spec. act. 52 units/mg protein. Therefore this procedure results in a purification factor of 346 and a yield of 46%. The yield resulting from the purification of adenylate kinase of *E. coli* strain CR341 was somewhat higher (58%) and the specific activity of this enzyme was 325 units/mg protein (purification factor 464). Fig.1 shows the purity of adenylate kinase prepared by this two-step procedure.

3.2. *Structural and kinetic properties of adenylate kinase of* E. coli

Adenylate kinase of *E. coli* is a relatively stable enzyme as compared to adenylate kinase of rabbit muscle or erythrocyte [1]. Only 8% (strain CR341) and 18% (strain CR341 T28) of enzymic activity were lost when purified *E. coli* adenylate kinase (10 μg/ml) was incubated at room temperature for 4 h. Moreover, no decrease in activity was observed when the pure enzyme (50 μg/ml) was incubated for 2 h with 1 mM DTNB in 50 mM phosphate (pH 7.4), as compared to the control (not shown). In this respect, *E. coli* adenylate kinase resembles liver, yeast and porcine heart acidic adenylate kinase [8,9,17]. After denaturation in 6 M GdmCl both mutant and parent adenylate kinase returned rapidly to the native, active state by simple dilution of GdmCl at 27°C. The renaturation yield was 75–100% for the parent adenylate kinase and 60–80% for the enzyme of the mutant strain. Since renaturation occurs even during spectrophotometric assay of adenylate kinase, it is possible to measure the rate of renaturation by following the absorption increase due to the formation of NADPH at different time intervals. From these data one can calculate the first order rate constant of the renaturation process (fig.2). In the case of adenylate kinase of the parent strain, $k_1 = 2.9 \times 10^{-2}\ s^{-1}$, whereas for the mutant enzyme $k_1 = 1.6 \times 10^{-2}\ s^{-1}$.

The affinity for nucleotides of purified adenylate kinase from parent and mutant strain

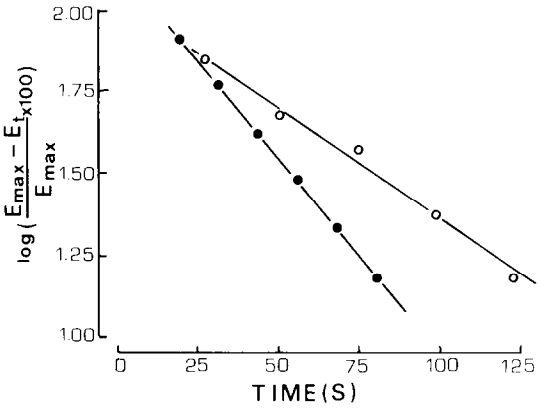

Fig.2. Kinetics of reactivation of *E. coli* adenylate kinase after denaturation by 6 M GdmCl. The 'renaturing' medium is the assay medium in the direction of ATP formation described in section 2. The final concentration of adenylate kinase (assuming M_r 27500) was 4.3 nM for the strain CR341 (●) and 7.4 nM for the strain CR341 T28 (○), respectively. E_t, the rate of absorption increase at 340 nm for different time values; E_{max}, the maximal rate usually attained 3–4 min after the start of the reaction. The activity of native adenylate kinase was constant during a time interval of at least 5 min.

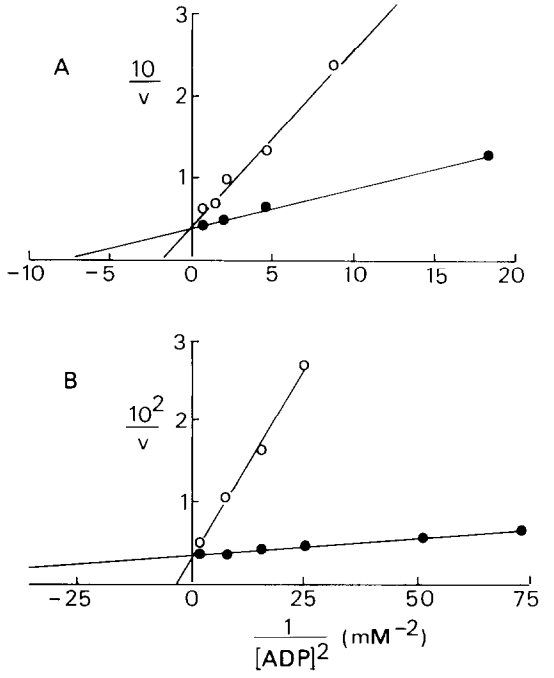

Fig.3. Inhibition of *E. coli* adenylate kinase by Ap_5A: (A) Enzyme from strain CR341 T28; (B) Enzyme from strain CR341; (●) No inhibitor; (○) 2.35 μM Ap_5A. The rate of adenylate kinase activity is expressed as μmol.min^{-1}.mg protein^{-1}.

were in good agreement with those in [10–12]. In addition, *E. coli* adenylate kinase was found to be strongly inhibited by Ap_5A (K_i = 0.6 μM for adenylate kinase of strain CR341 and 2.1 μM in the case of mutant enzyme, fig.3). Our data are at variance from those in [18] which showed a very weak inhibition by Ap_5A of a crude preparation of adenylate kinase from *E. coli*, strain MRE600. Since this nucleotide appeared to be stable when added to various cell extracts, including *E. coli* MRE600 [18], it is possible that adenylate kinase from different strains of *E. coli* exhibits different sensitivities towards Ap_5A.

4. DISCUSSION

The isolation of a thermosensitive mutant of *E. coli* [10,13,19,20] with a thermolabile adenylate kinase and a thermolabile glycerol-3-phosphate acyl transferase (both labilities apparently resulting from a single mutation) [21,22] focused attention on the functional relationship of these enzymes both in vivo and in vitro [23,24]. In [12] adenylate kinase was purified from *E. coli* strains CR341 and CR341 T28 mainly for kinetic comparisons; therefore, efforts to maximize yield (5%) did not seem necessary. In [24] wild-type adenylate kinase from *E. coli* was reported purified to homogeneity, but the enzyme was not purified from the mutant strain using the same protocol. The purification procedure described in this work has several obvious advantages:

(i) The procedure is fast (24 h), requiring only two chromatographic steps and allows purification of both wild-type and mutant adenylate kinase.

Streptomycin sulfate and ammonium sulfate precipitations, steps that commonly precede chromatography [10–12,24], were found to be useless. Their elimination improves the yield substantially and shortens the exposure of mutant adenylate kinase to proteases, which probably are involved in the partial inactivation of the mutant enzyme during purification;

(ii) The elution of adenylate kinase from the crude extract after its binding to blue–Sepharose could be achieved with natural nucleotides [12,24] but much more specifically with Ap_5A.

When large amounts of bacteria (> 50 g wet wt) are processed, an additional step can be used to minimize the consumption of Ap_5A, an expensive reagent. If, in the first step the adenylate kinase from the crude extract is eluted with 1 mM ATP and 1 mM AMP after its binding to blue–Sepharose at pH 7.4, a purification factor of 25 is obtained. After gel filtration on Sephadex G-75 or Sephadex G-100, a second chromatography on blue–Sepharose (small column of 2–5 ml) with Ap_5A elution yields pure enzyme with a final yield between 30% (mutant adenylate kinase) and 48% (wild-type enzyme);

(iii) The procedure should find application to the purification of ^{35}S-labeled adenylate kinase from 200–500 ml bacterial cultures (~0.4–1.0 g wet wt) and could help to further study the thermolability of mutant enzyme especially in crude extracts (in preparation).

ACKNOWLEDGEMENTS

This work was supported by grants from the Centre National de la Recherche Scientifique (Laboratoire Associé no. 270); the Fondation pour la Recherche Médicale, Institut National de la Santé et de la Recherche Médicale, and the Délégation Générale à la Recherche Scientifique et Technique. We thank A. Ullmann for interest and critical comments, A. Danchin for bacterial strains and fruitful discussion, and K. Pepper for carefully reading this manuscript.

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