Cloning and sequencing of the adenylate kinase gene (adk) of Escherichia coli

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ABSTRACT

Adenylate kinase, the product of the adk locus in Escherichia coli K12, catalyzes the conversion of AMP and ATP to two molecules of ADP. The gene has been cloned by complementation of an adk temperature sensitive mutation. The DNA sequence of the complete coding region and of 5'- and 3'-untranslated regions were determined. The resulting protein sequence was found to contain several regions of high homology with cytosolic adenylate kinase of pig muscle (AK1), whose three-dimensional structure has been determined. The most significant of the amino acid exchanges is the replacement of histidine 36 with glutamine. This residue is believed to play a role in catalysis through metal ion binding. The codon usage pattern and the determination of adenylate kinase molecules per cell shows that the enzyme is one of the more abundant soluble proteins of the bacterial cells.

INTRODUCTION

Adenylate kinases catalyse the reaction: MgHTP + AMP → MgNDP + ADP (N=A,G). They are small ubiquitous enzymes with at least three isoforms (AK1, AK2 and AK3) ranging in Mr between 22 000 for AK1 and 30 000 for AK2 (for a review see [1]), which are important for growth and maintenance of probably any living organism [2]. In Escherichia coli, by using temperature sensitive mutants, it has been found that the enzyme is essential for growth [3].

The three-dimensional structure of the cytosolic porcine AK1 has been determined to high resolution [4,5] and it is generally assumed that the polypeptide chain tracing of all adenylate kinases around the active site(s) is identical [6,7]. No three-dimensional structures of enzyme-ligand complexes have been reported so far. Soaking of crystals with substrates and substrate analogues have only led to the putative assignment of two adenine binding pockets. The involvement in ligand binding of any side chain residues as deduced from biochemical and X-ray studies and sequence comparisons [8,9] is thus presently very tentative. Our aim is to use site directed mutagenesis to replace various amino acid residues which have been
postulated to be involved in the enzyme-substrate-interaction. To do this, we have cloned and sequenced the \textit{adk} gene of E.coli situated at 11 min on the E.coli map [10].

\section*{MATERIALS AND METHODS}

\subsection*{Bacterial strains:}
Wild type E.coli K12 strain 1100 (F-, \textit{supE}, \textit{andA100}), from which the gene library was constructed, was kindly provided by H. Hoffmann-Berling. Strain DH1(\textit{ACh616}) is a \textit{\lambda} lysogen derivative [11] of strain DH1 [12] and was obtained from K. Geider. The \textit{adk} temperature sensitive strain CV2 (\textit{topA22, phoAB, adk2, smf627, fasL701, relA1, gloR2, pit10, spoT1, \textit{T2}R}) [13] was obtained from the E.coli Genetic Stock Center, Yale University, New Haven through B. Bachmann. Wild type strain MRE 600 has been described [14]. Strain JM 101 [15] for M13 sequencing and minicell strain DS410 [16] were provided by T. F. Meyer.

\subsection*{Construction of gene library}
DNA of strain 1100 was isolated and purified by phenol extraction. After digestion with restriction enzymes Sau3A and HpaII, DNA fragments of approximately 3-5 kb length were obtained from the digestion mixture by performing a sucrose gradient centrifugation. pBR322 was digested with ClaI and HindIII, the 6bp nucleotide was removed by centrifugation desalting, after which the vector was further digested with BamHI, as described [17]. Vector and fragments were phenol extracted, ligated and transformed into DH1(\textit{ACh616}) by the method of Hanahan [12] such that 60 000 independent ampicillin resistant clones were obtained. The cells were scraped off the plates and plasmid DNA was isolated by the Triton-X method described by Maniatis et al. [18], and stored as an ethanol precipitate at -70°C.

\subsection*{Cloning of the \textit{adk} gene}
CV2 was transformed with an aliquot of the gene library DNA described above according to the method of Hanahan[12]. Most of the transformation mixture was incubated on ampicillin (50ug/ml) plates at 42°C, a small portion also at 28°C to determine the total number of transformants. From the clones obtained at 42°C, plasmid DNA was isolated from 1ml mini-cultures and their size determined. For activity measurements, cell free extracts were prepared from 1ml overnight cultures. The cell pellet was suspended in 500uL lysis buffer (50 mM Tris/HCl pH 7.4, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and 0.5 mM dithiothreitol) and 3ul of freshly prepared lysozyme solution (10mg/ml) was added. After 10-20min at room temperature
3ul of a sodium deoxycholate solution (40 mg/ml) was added and the reaction was incubated for another 15 minutes. After centrifugation, the supernatant was taken off and an aliquot of it was used in the assay for adenylate kinase activity.

Adenylate kinase assay and number of molecules per cell

Adenylate kinase activity was determined by a coupled optical test of the reverse reaction (2ADP → AMP + ATP): To a mixture of 50 mM Tris/ HCl pH 7.6, 50 mM KCl, 5 mM MgCl₂, 10 mM glucose, 2 mM ADP, 0.5 mM NADP⁺, and 0.3U of both hexokinase and glucose-6-phosphate-dehydrogenase, an aliquot of the bacterial extract was added and the absorption increase at 340nm due to an increase of NADPH was followed.

For the estimation of the number of adenylate kinase molecules per cell, the volume activities obtained above were divided by the specific activity of the E.coli adenylate kinase, which has been determined for purified enzyme to be 15800 (Molxmol⁻¹xmin⁻¹)[19] to obtain the number of enzyme molecules. This was related to the number of cells, determined as the viable cell count by plating out dilutions of the cell culture on appropriate plates.

In vitro transcription and translation in minicells

Minicell producing strain DS410 was transformed with two plasmids of adk positive clones. Minicell suspension was prepared as described [20] and incubated with an amino acid mixture containing 16.5uCi [³⁵S]methionine. Minicells were collected by centrifugation and dissolved in protein sample buffer (62.5mM Tris.HCl pH6.8, 2% sodium dodecylsulfate, 10% glycerol, 0.5% dithiothreitol and 0.001% bromphenolblue). HCl precipitable counts were determined on Whatman filter paper and an aliquot of the solution was electrophoresed on a 15% polyacrylamide gel following the Laemmli protocol [21]. The gel was dried and autoradiographed. ¹⁴C-labelled marker proteins were run in separate lanes.

DNA Manipulations

All DNA manipulations were done as described in the Laboratory Manual of Maniatis et al. [18]. DNA sequences were determined by both the base specific chemical cleavage method of Maxam and Gilbert [22] and the dideoxy chain termination method of Sanger et al. [23] using M13mp8 and M13mp9 phages [24].

Enzymes and Chemicals

Restriction enzymes were obtained from Boehringer Mannheim and Bethesda Research Lab., alkaline phosphatase from Boehringer Mannheim, T₄
polynucleotide kinase and T4 DNA ligase from Bethesda Research Lab., and DNA polymerase I (Klenow fragment) from Renner (Heidelberg). Enzymes used in optical test were from Boehringer Mannheim, Lysozyme from Serva (Heidelberg). Radiochemicals [γ-32P]dATP, 35S-methionine and radioactive marker proteins were obtained from New England Nuclear. For sequencing according to Maxam and Gilbert [22] a complete sequencing kit from New England Nuclear was used. All other chemicals were of the highest purity available.

RESULTS

Cloning and clone characterisation

A gene library from E.coli strain 1100 was constructed in ClaI and BamHI digested pBR322 using limited Sau3A and HpalI digestion of chromosomal DNA and size fractionation of fragments as described in Methods. More than 60% of colonies contained inserts of sufficient size. An aliquot of the library was transformed into CV2, which is a temperature sensitive mutant for adk, the gene for adenylate kinase [13,25,26], and grown on ampicillin plates at

<table>
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<tr>
<td></td>
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<td>0.5</td>
</tr>
<tr>
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<tr>
<td></td>
<td>b 0.6</td>
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<tr>
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<td></td>
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<tr>
<td></td>
<td>b 140.0</td>
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<tr>
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</tr>
<tr>
<td>CV2(pAK60)</td>
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Enzyme activities in units per ml cell culture (a) and estimation of numbers of molecules per cell in thousands (b) were obtained as described in Methods. ON is an overnight culture.
42°C. 60 colonies which grew at the nonpermissive temperature (from an estimated 300,000 growing at 28°C) were isolated. The plasmid size of these 60 clones varied between 7 and 12 kb corresponding to an insert size between 3 and 8 kb. Adenylate kinase activities of those 60 clones measured by an

Figure 1: Autoradiogram of 35S-labelled proteins obtained by coupled transcription/translation of plasmids in minicells. Plasmids pAK17 (lanes 1,3) and pAK60 (lanes 2,4) were transformed into minicell producing strain DS410, labelled with 35S-methionine, and separated on polyacrylamide gel, using 70000 (lanes 1,2) or 140000cpm (lanes 3,4) as described in Methods. C-labelled marker proteins (and their molecular weight) are: trypsin inhibitor (20100), lane 5; carbonic anhydrase (29000), lane 6; egg albumine (46000), lane 7. Different exposure times were used for lanes 5-7.
Figure 2: Restriction map of plasmids pAK60 and pAK17 and constructions of pAK601 and pAK171.
The gene for ampicilline resistance (AmpR) on pBR322 is marked by an arrow. Thick lines represent the inserted chromosomal DNA. Abbreviations for restriction enzymes are: C= ClaI; E= EcoRI; H= HindII; P= PstI; Pv= PvuII; Sa= SalI; Sm= SmaI. Restriction sites of ClaI(AC) and BamHI(AB) of pBR322, between which the DNA fragments have been inserted, are also shown. pAK601 and pAK171 were obtained by digesting pAK60 and pAK17 with SalI and ClaI (underlined) and ligation the large fragments after generation of blunt ends.
optical test of the reverse reaction were on an average approximately 100 times higher than the activity in CV2 and 20 times higher than in wild type MRE600. The adenylate kinase activity of CV2(pAK50) is 60fold over the activity found in wild type MRE600 and can thus be used to greatly simplify the purification of the E.coli enzyme [19,27]. Four clones, pAK16, 17, 32 and 60, which had appropriate size and adenylate kinase activities as shown in Fig.2 and Table1, were chosen for further analysis.

Fig.1 is an autoradiogram of the $^{35}S$-labelled proteins obtained from in vitro transcription and translation of pAK17 and pAK60 in the minicell system. The very strong band at Mr 27000 has the apparent molecular weight reported for adenylate kinase of E.coli [19,27,26] and is thus a strong indication that the whole structural gene is present at least on those two plasmids.

The coding region for adenylate kinase is expected, on the basis of the reported Mr of the E.coli enzyme, to be approximately 700 nucleotides long. Since the four clones, two of which are shown in Fig.2, contained much larger inserts ranging between 2.5 and 5.5 kb, a restriction analysis was performed in order to find a region which is common to all four plasmids. Fig.2 shows the restriction maps for the plasmids pAK17 and pAK60. A common feature is a region between the restriction sites of SmaI and PvuII situated in the vector in two different orientations which could possibly contain the AK coding region. To confirm our assignment, deletions were introduced both into pAK17 and pAK60, as shown in Fig.2, and these constructions were transformed into CV2. Transformants at 42°C were only obtained for the deletion in pAK60 (pAK601), not for the deletion in pAK17 (pAK171). Since also the adenylate kinase activity in the cell free extract was not affected
Figure 4: DNA sequence of adk and amino acid sequence of E.coli adenylate kinase.
Only the sequence of the sense strand is shown. Numbering of DNA sequence starts at PvuII restriction site at the 5' end. The arrows above the sequence indicate the open reading frame on the sense strand, the arrows below indicate the length of the open reading frame on the antisense strand, which is not shown. Underlined are possible -35 and -10 transcription control elements. Overlined is a possible Shine and Dalgarno sequence [34].
in going from pAK60 to pAK601 we can conclude that the adk gene is located between the ClaI restriction site and the EcoRI restriction site of pBR322. Within this region there are restriction sites of PvuII, EcoRI, HindII and Sau3A, which can be used for DNA sequencing.

**DNA and derived protein sequence**

Fig. 3 shows the sequencing strategy used and Fig. 4 the sequence obtained following the method of Maxam-Gilbert [22] and the method of Sanger et al. [23]. Thus we have obtained 193 bp of 5'-untranslated, the 642 bp of the coding region and 220 bp of 3'-untranslated region of the adk gene (Fig. 4). Although the open reading frame on this sense strand of the DNA extends 72 bp upstream into the 5'-direction, we feel certain that the adk structural gene starts at the methionine codon, bp 194, for the following reasons. 1. There is no methionine codon further upstream within the open reading frame. 2. If translation would start at the rarely used valine codon GTG, the resulting adenylate kinase would be 19 amino acids longer. Thus the E.coli protein should be as large as AK2, the enzyme from the outer compartment of mitochondria, whereas according to gel electrophoresis data AK2 should be larger than the E.coli enzyme [19,27,28]. The molecular weight of the protein as proposed here would be 23500, which is in accord with the molecular weight as determined by SDS polyacrylamide electrophoresis (Fig. 1), because for many adenylate kinases one finds that the molecular weight by SDS gel electrophoresis is generally overestimated by approximately 3000 daltons [H. Schirmer, personal communication].

Interestingly, there is another open reading frame of 733 bp on the other DNA strand which is longer than the ORF of the adk gene and is indicated also in Fig. 4 by arrows. The corresponding protein sequence is not related to adenylate kinase. To find out whether this hypothetical protein has any resemblance to other proteins, its sequence was compared to the 3061 proteins of the PIR (NBRF) Data Base using the program HAXHOM of C. Sander based on the algorithm by Smith and Waterman [29]. We find no statistically significant homology. Thus we cannot speculate on the possible significance of this result.

**Codon usage and abundance of adenylate kinase**

Codon usage of the adk gene is shown in Table 2, which in general resembles that of a highly expressed protein [30]. It is worth noting that in a few instances there is a very selective codon usage, i.e. there is only CCG used for proline and only UUC for phenylalanine. This is similar to the codon usage pattern of EF-Tu [31,32] and more selective than that of i.e.
Table 2: Codon usage and amino acid composition of E.coli adenylate kinase

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<td>TAG 0</td>
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<td>Gly 20</td>
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Total: 214 Amino acids; Molecular weight: 23559

the ribosomal proteins [30]. This suggests, that adenylate kinase is one of the more highly expressed proteins in E.coli.

We have estimated the number of adenylate kinases per cell using the volume activities shown in Table 1, which are determined in the crude extract of various bacterial cultures in various growth phases. Using these values and the reported specific activity of E.coli adenylate kinase [19], we obtain the total number of enzyme molecules, which are then related to the number of cells. Using this calculation we find that in wild type E.coli there are approximately 10000 copies per cell, see Table 1. This means that this enzyme is as abundant as the ribosomal proteins or the aminocacyl-tRNA-synthetases [33]. Not surprisingly, the number of molecules per cell is also growth rate dependent and is highest in rapidly dividing cells, since the enzyme has to replenish the ATP pool. The amount of adenylate kinase in CV2(pAK601) is much higher than in CV2 or wild type HRE600, but relatively
Figure 5: Sequence comparison between E.coli adenylate kinase and adenylate kinase 1 from pig muscle [35].

Sequence comparison was performed using the MAXHOM program of C.Sander which is based on the algorithm of Smith and Waterman [29]. Identical amino acids are marked by asterisks, homologous regions are underlined. Deletions are marked by points.

The untranslated regions of the adk gene were studied with the aim of identifying possible promoter and translation control elements. There is a purine rich region in front of the translation start point, overlined in Fig.4, which could serve as ribosomal binding site as defined by Shine and Dalgarno [34], since it has an optimal distance of 8bp to the ATG start codon. We have tentatively assigned a -10 and -35 region in the sequence, underlined in Fig.4, as the possible promoter for the adk gene, purely on the basis of sequence homology to the consensus promoter sequence and relative distance to each other and to the possible transcription initiation startpoint. There is no other similar fit to the consensus promoter sequence.
in the 5'-untranslated region of the sequence. The fact that the cloning of the \textit{adk} gene leads to overexpression of the adenylate kinase seems to indicate, that the protein is expressed from its own promoter.

The amino acid sequence of E.coli adenylate kinase is compared in Fig. 5 to AKI [35], the pig heart cytosolic enzyme, whose three-dimensional structure has been determined. Several other sequence comparisons have been reported [6,7]. They all show preserved regions of homology which are situated around residues 14-24, 36-47, 88-101 and 148-168, taking the position numbers from the AKI sequence [35]. These stretches of homology can be assigned to various structural features of the three-dimensional structure, which together are believed to form the scaffold for the AMP and MgNTP nucleotide binding sites. Adenylate kinase of E.coli, which is shorter than AKI at the N- and C-terminus, but has internal extra sequences around residues 120-140, has an overall homology to AKI of 36%. It shows the same regions of homology, where many residues are totally conserved between various adenylate kinases. All the residues, that have been implicated to be involved in nucleotide binding are conserved except histidine 36, which in E.coli adenylate kinase is replaced by glutamine (residue 28). If histidine is indeed complexed to the metal ion as has been suggested [36,37,38], it is conceivable, that the side chain of glutamine performs the same function and it would be interesting to test, what effect, if any, the replacement of glutamine by histidine in E.coli adenylate kinase might have.

So far no structure determinations of an enzyme-ligand-complex have been reported. The contact points between polypeptide chain and nucleotides have been proposed only on the basis of soaking experiments and biochemical evidence [8]. However, the conclusions reached are disputed because of seemingly contradictory evidence coming from NMR experiments [38]. The fact that now the gene for adenylate kinase is available and that the protein for which it codes is overproduced in these clones, should allow us to check these predictions about enzyme-substrate interactions by structural and kinetic investigations of mutants produced by site-directed mutagenesis.

REFERENCES