Conservative Replacement of Methionine by Norleucine in *Escherichia coli* Adenylate Kinase

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*Escherichia coli* grown in limited methionine and excess norleucine media accumulate cyanogen bromide-resistant species of proteins after the methionine supply is exhausted. Bacteria, transformed by recombinant plasmid pIPD37 carrying the *adk* gene and grown under limiting methionine and excess norleucine, synthesize 16–20% of adenylate kinase molecules having all 6 methionine residues replaced by norleucine. Species showing only partial replacement of methionine residues by norleucine are identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after cyanogen bromide treatment of pure enzyme.

Norleucine-substituted adenylate kinase shows structural and catalytic properties similar to the wild-type protein as indicated by circular dichroism spectroscopy and kinetic experiments but exhibits a much higher resistance to hydrogen peroxide inactivation under denaturing conditions.

In vivo replacement of naturally occurring amino acids by their analogs offers a convenient and powerful tool for probing the effect of molecular alterations on the biological activity of proteins (1). The methionine analog norleucine is known to increase the culture yield of methionine auxotroph strains of *Escherichia coli* (2, 3), although it cannot alone sustain the growth of such auxotrophs (3), as does selenomethionine (4). Sterically superimposable to methionine, norleucine lacks a sulfur atom and is thus unable to ensure the metabolic functions of methionine, via the formation of S-adenosylmethionine. On the other hand, norleucine-containing proteins resemble their wild-type counterparts, as demonstrated for *E. coli* β-galactosidase (5) and for staphylococcal nuclease (6).

In search of new methods for obtaining complementary fragments of *E. coli* adenylate kinase able to reconstitute catalytically active species (7, 8), we investigated the effect of in vivo substitution of methionine residues by norleucine. Since norleucine is incorporated randomly in place of methionine (3), we expected a large variety of adenylate kinase species containing from 1 to 6 methionine residues replaced by norleucine. Some of these species might be easily identified by resistance to CNBr cleavage and tested for their catalytic properties or their ability to interact with nucleotides.

In this paper we show that *E. coli* transformed by recombinant plasmid pIPD37 carrying the *adk* gene and grown in the presence of an excess of DL-norleucine (1.5 × 10⁻⁶ M) over l-methionine (2 × 10⁻⁶ M) synthesizes a large proportion (between 16 and 20%) of active adenylate kinase which is insensitive to CNBr cleavage. We also examined the consequences of this amino acid replacement on the stability of adenylate kinase in the presence of chemical reagents such as oxidants.

**EXPERIMENTAL PROCEDURES**

Chemicals—Adenine nucleotides, substrates, and coupling enzymes were from Boehringer Mannheim (a generous gift of Professor F. H. Schmidt (University of Heidelberg)). TPCK-trypsin and soybean trypsin inhibitor were from Sigma. Blue-Sepharose was obtained from Pharmacia LKB Biotecnologies Inc. Urea (fluorimetrically pure) was from Schwarz/Mann. [α²P]H₃PO₄ (carrier-free) and L-[³⁵S]methionine (1300 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, United Kingdom). BzATP was synthesized as described by Williams and Coleman (9). [γ-³²P]BzATP was prepared according to Glynn and Chappell (10). The identity of the compound was checked by thin layer chromatography on cellulose or polyethyleneimine-cellulose, from its absorption spectrum (ε₂₈₀ = 41) (11) and substrate capacity for yeast phosphoglycerate kinase (good) and hexokinase (poor). Photoreactivity was ascertained as described by Williams and Coleman (9).

Bacterial Strains and Culture Conditions—The strain PMX used in this work is a derivative of Hfr P4X8 bearing a metB mutation from the Pasteur Institute collection (12). The methionine auxotrophy conferred by this mutation does not revert in standard genetic assays (13) and was not analyzed further. Following conventional procedures (14), strain PMX was transformed with plasmid pIPD37 (kindly provided by Dr. Isabelle Saint Girons (Pasteur Institute, Paris)), a derivative of pBR322 carrying the *E. coli adk* gene (7) yielding strain PMX.

The strain PMX was grown in a 20-liter Chemap fermenter filled with 15 liters of synthetic medium (pH 7) having the following composition (g/liter): KH₂PO₄, 6; K₂HPO₄, 18.3; (NH₄)₂SO₄, 4; MgSO₄·7H₂O, 0.4; FeSO₄·7H₂O, 5 × 10⁻⁴; glycerol, 8; ampicillin, 0.1; L-methionine, 3 × 10⁻⁴ (2 × 10⁻⁴ M); dl-norleucine, 0.2 (1.5 × 10⁻⁴ M). The culture was heated at 37 °C and oxygenated with an air flow of 10 l/min under vigorous agitation. When a predetermined value of turbidity was attained, a fraction (0.1–3 liters) of the culture was withdrawn and immediately centrifuged at 5,000 × g for 30 min at 4°C. The resulting pellet was washed and resuspended either with 50 mM Tris-HCl (pH 7.4) or with 50 mM ammonium bicarbonate (pH 7.8) and disrupted by sonication at 20 KHz and 100 watts (3 × 4

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1 The abbreviations used are: CNBr, cyanogen bromide; BzATP, 3’-O-(4-azoniobenzyl)adenosine 5’-triphosphate; SDS, sodium dodecyl sulfate; TPCK, L-1-tosylamido-2-phenyl-ethylchloromethyl ketone; Blue-Sepharose, Cibacron Blue G-3A Sepharose CL-6B.
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min). Cell debris were removed by centrifugation at 13,000 × g for 20 min at 4 °C.

Purification of Adenylate Kinase and Assay of Enzymatic Activity—Adenylate kinase from the overproducing strain was purified as described previously (7, 15), by a two-step procedure involving chromatography on Blue-Sepharose and gel permeation chromatography on Ultragel ACA 54. After extensive dialysis against several changes of 50 mM ammonium bicarbonate, the enzyme solution was lyophilized and was cyanylated according to Jacobson et al. (16). The cyanylated protein was cleaved and the resulting peptides (C1, residues 1-76; C2, residues 77-214) purified under denaturing conditions as described previously (7). Thermosensitive adenylate kinase from E. coli strain CR341T28 was purified according to Gilles et al. (17).

Adenylate kinase activity was determined at 27 °C in a final volume of 1 ml using a spectrophotometric assay system both in the direction of ATP (forward reaction) and of ADP (reverse reaction) formation (7). One unit of enzyme activity corresponds to 1 μmol of product formed/min.

Chemical Cleavage of Adenylate Kinase with Cyagen Bromide—Pure adenylate kinase or E. coli extracts in ammonium bicarbonate were lyophilized, then dissolved in a solution of 2.5% CNBr in 70% formic acid (200 μl for each milligram of protein). After 24 h of incubation at room temperature in the dark, samples were diluted 5-fold with twice distilled water and lyophilized.

Circular Dichroism Measurements—The CD spectra were recorded with a Jobin Yvon Mark IV dichrograph connected to a Digital microcomputer. Enzyme was solubilized in 5 mM ammonium bicarbonate (pH 7.8). Measurements were performed in quartz optical cylindrical cells with 0.1-cm path length, for the spectral range from 260 to 185 nm. Results are expressed in mean residue molar ellipticity, θ, in degrees-cm²-dmol⁻¹. For estimation of secondary structure, CD curves in the 190-260 nm range were processed by the method of Chen et al. (18).

Analytical Procedures—Proteins were measured according to Bradford (19) with purified adenylate kinase (A/66 = 5.0) as the calibration standard. Amino acid analysis after hydrolysis of proteins at 110 °C with 6 N HCl for 24 h was performed on a Biotronik LC 5001 amino acid analyzer. Equilibrium dialysis experiments and SDS-polyacrylamide gel electrophoresis were performed as described in previous publications (7, 15, 17, 20).

RESULTS

Cyanogen Bromide-Resistant Species of Adenylate Kinase Accumulate after Methionine Depletion in the Growth Medium—Preliminary experiments with methionine auxotrophs grown on limiting L-methionine (3 to 5 × 10⁻⁴ M) showed that growth ceased when the methionine supply was exhausted. With 30-50-fold molar excess DL-norleucine over L-methionine, there was an additional growth of bacteria corresponding to an optical density at 570 nm of 0.18 (lanes A and C), 0.3 (lane D), 0.4 (lane E), 0.6 (lane F), and 0.7 (lanes B and G) were treated (lanes C-G) or not (lanes A and B) with CNBr; then examined on a 12.5% SDS-polyacrylamide gel and stained with Coomassie Blue. Lane S, standard proteins, from top to bottom: a, phosphorylase a (94,000); b, bovine serum albumin (67,000); c, ovalbumin (43,000); d, carbonic anhydrase (30,000); e, soybean trypsin inhibitor (20,100); f, lysozyme (14,000). Arrows situated on the right side of the figure indicate the position of adenylate kinase and of peptide corresponding to residues 97-214 (resistant to CNBr cleavage since its Met14 is followed by a Thr residue).

FIG. 1. Synthesis of CNBr-resistant species of adenylate kinase shows the electrophoretic pattern of adenylate kinase purified on Ultrogel AcA 54. After extensive dialysis against several changes of 50 mM ammonium bicarbonate, the enzyme solution was lyophilized and was cyanylated according to Jacobson et al. (16). The cyanylated protein was cleaved and the resulting peptides (C1, residues 1-76; C2, residues 77-214) purified under denaturing conditions as described previously (7). Thermosensitive adenylate kinase from E. coli strain CR341T28 was purified according to Gilles et al. (17).

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Adenylate kinase specific activity, determined in cell extracts of E. coli harvested at different phases of growth in limited methionine and excess norleucine media, shows little variation within the limit of experimental errors. However, the species resistant to CNBr accumulate only in the late phase of growth. In bacteria from the stationary phase, between 16 and 20% of total adenylate kinase activity was found resistant to CNBr cleavage (Fig. 1A). Since enzyme expressed by the pLPD37 plasmid represents 12-15% of total E. coli proteins, SDS-polyacrylamide gel electrophoresis of crude extracts before and after treatment with CNBr showed accumulation of some species having presumably all methionine residues replaced by norleucine (Fig. 1B). Gel analysis was in good agreement with adenylate kinase activity assays.

Amino Acid Composition of Adenylate Kinase Purified at Different Stages of Growth in Excess Norleucine Medium—During the early phase of growth, E. coli accumulated mainly methionine-containing species of proteins, so that only 5% of the methionine residues were substituted by norleucine in purified preparations of adenylate kinase (Table I). At the stationary phase, half of the methionine residues of adenylate kinase were found replaced by norleucine. This suggests that only adenylate kinase species having all of the 6 Met residues replaced by norleucine (with the possible exception of the N-terminal Met) remain active after CNBr treatment. Fig. 2A shows the electrophoretic pattern of adenylate kinase purified from bacteria grown in presence of an excess of norleucine and cleaved with CNBr. Adenylate kinase treated with CNBr yielded several polypeptide bands in SDS-polyacrylamide gel electrophoresis. The dominant bands corresponded to the uncleaved enzyme and to a polypeptide fragment 97-214 (resistant to CNBr cleavage since its Met14 is followed by a Thr residue).

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enzyme as deduced from the nucleotide sequence of the ary phase of growth, treated with CNBr then with trypsin under protection with ATP. Uncleaved protein was separated from fragments by trypsin, especially when ATP was present in the incubation medium. All polypeptides resulting from partial cleavage of adenylate kinase with CNBr were rapidly proteolyzed by trypsin in less than 10 min at 30°C, whereas reconstituted protein incorporated [γ-32P]Bz2ATP, thus indicating that the isolated C1 and CP peptides are completely digested by Bz2ATP. As shown in Fig. 3, we wanted to know if the polypeptides resulting from incomplete CNBr cleavage of the adenylate kinase are very similar (Fig. 4) showing that norleucine plays the same role as methionine in maintaining the intact secondary structure of the protein. The composition in the secondary structure of norleucine-containing adenylate kinase obtained by processing CD curves in the near UV gave 46% α-helix, 14% β-sheet, and 40% remainder, as compared to 50% α-helix, 15% β-sheet, and 35% remainder for methionine-containing adenylate kinase.

Isolation of Adenylate Kinase Species Containing Norleucine in Place of All Methionine Residues—As norleucine is randomly incorporated at methionine sites (3), it might seem difficult to isolate a homogenous population of molecules. However, in the particular case of adenylate kinase, all species having even a single methionine residue gave fragments highly sensitive to proteolysis by trypsin after an initial CNBr treatment (Fig. 2B). Intact molecules of adenylate kinase, presumed to contain all 6 methionine residues replaced by norleucine and resistant to trypsin digestion, were thus purified by rechromatography on Blue-Sepharose. Amino acid analysis of adenylate kinase repurified after CNBr cleavage and trypsin treatment (column D in Table I) confirmed the total replacement of methionine by norleucine.

Structural and Catalytic Characteristics of Norleucine-containing Species of Adenylate Kinase from E. coli—The CD spectra of methionine-containing and norleucine-containing adenylate kinase are very similar (Fig. 4) showing that norleucine plays the same role as methionine in maintaining the intact secondary structure of the protein. The composition in the secondary structure of norleucine-containing adenylate kinase obtained by processing CD curves in the near UV gave 46% α-helix, 14% β-sheet, and 40% remainder, as compared to 50% α-helix, 15% β-sheet, and 35% remainder for methionine-containing adenylate kinase.

Determination of the kinetic parameters of norleucine-containing adenylate kinase showed a 20% decrease in maximal catalytic activity, (326 μmol/min·mg of protein in the forward reaction) whereas affinity for nucleotides was practically unaffected. Thus, the apparent $K_m^b$ was 105 μM for

### Table I

Amino acid composition of E. coli adenylate kinase purified at different phases of growth in restricted methionine and excess norleucine medium

| Amino acid | A | B | C | D | E |
|-----------|---|---|---|---|---|
| Cys       | 1.1| 1.4| 1.3| 1.2| 1  |
| Asx       | 21.6| 21.8| 21.7| 22.0| 21 |
| Thr       | 9.8| 9.7| 10.5| 9.7| 11 |
| Ser       | 4.6| 4.6| 4.8| 4.5| 5  |
| Glx       | 26.0| 25.6| 25.9| 25.8| 26 |
| Pro       | 8.9| 8.7| 10.5| 8.1| 10 |
| Gly       | 20.1| 20.0| 19.4| 21.5| 20 |
| Ala       | 19.0*| 19.0| 19.0| 19.0| 19 |
| Val       | 17.9| 18.1| 19.4| 18.0| 19 |
| Met       | 4.7| 3.1| 3.1| 0.6| 6  |
| Ile       | 12.5| 12.8| 14.2| 13.8| 14 |
| Leu       | 14.9| 15.9| 17.3| 16.8| 16 |
| Tyr       | 5.6| 5.1| 6.9| 5.5| 7  |
| Phe       | 4.8| 4.6| 5.4| 4.8| 5  |
| His       | 3.3| 3.1| 3.5| 3.8| 3  |
| Lys       | 15.6| 17.1| 18.9| 17.4| 18 |
| Arg       | 11.5| 12.6| 15.3| 13.0| 13 |
| Trp       | ND| ND| ND| ND| 0  |
| Norleucine| 0.3| 2.5| 2.9| 5.9| 5  |

* Arbitrarily taken as reference value for $M_r = 23,500$.

** Not determined.

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norleucine-containing adenylate kinase, whereas methionine-containing enzyme had an apparent $K_m^{\text{ADP}}$ of 92 $\mu$M.

A characteristic of E. coli adenylate kinase is that AMP above 0.3 mM, but not 2' dAMP or other nucleoside mono-

phosphates, inhibits enzyme activity (22). This intriguing property can be interpreted as the result of competition between excess AMP and ATP for the nucleotide donor site of adenylate kinase. However, the apparent $K_m^{\text{ATP}}$ determined at optimal (0.3 mM) and inhibitory concentrations (4 mM) of AMP does not reveal significant differences (48 and 54 $\mu$M, respectively). Norleucine-containing adenylate kinase shows the same sensitivity to inhibition by excess AMP (more than 50% inhibition at 4 mM, taking the activity at 0.3 mM AMP as 100%) (Fig. 5). It is interesting to note that thermostable adenylate kinase (Pro$^{\text{7}}$→Ser) (17) is not inhibited by excess AMP, in agreement with data reported using partially purified enzyme preparations (22).

Hydrogen peroxide is known as a powerful methionine-oxidizing agent (23, 24). We thus examined its effect on methionine- and norleucine-containing adenylate kinase. In the presence of 0.1 M H$_2$O$_2$, under nondenaturing conditions, adenylate kinase activity remained unchanged for several hours at room temperature. In the presence of 6 M urea and 0.05 M H$_2$O$_2$, methionine-containing adenylate kinase was...
90% inactivated within 20 min at 19 °C (Fig. 6). The kinetics of inactivation showed two distinct steps. The rapid phase of inactivation corresponded to methionine oxidation to its sulf oxide, whereas the slow phase probably corresponded to oxidation of the cysteine residue. Under identical experimental conditions, norleucine-containing adenylate kinase was inactivated at a rate which corresponded to the slow phase inactivation of methionine-containing enzyme. When excess β-mercaptoethanol was added to protein in H2O2-urea medium after complete inactivation, full enzyme activity was restored within several minutes both in the case of methionine- or norleucine-containing adenylate kinase.

**Discussion**

Evidence has been accumulating from molecular genetics and protein engineering that proteins are largely impervious to point changes. Thus, the vast majority of β-galactosidase missense mutants show only a small loss of activity (25), and many residues at invariant sites of homologous proteins can be changed without causing much damage (26, 27). This robustness has been interpreted as a built-in anticipation of close sequence variants of a protein arising either by translation errors (25) or by point mutations (27). When incorporated into the bulk of cellular proteins amino acid analogs afford the phenotypic equivalent of many simultaneous mutations of a unique type. The fact that bacteria built with an artificial, albeit structurally conservative analog, remain viable and their proteins functional widens these current hypotheses of protein robustness (27, 28).

Thus, norleucine-substituted adenylate kinase of E. coli shows catalytic and structural properties close or identical to the wild-type protein. Our results are consistent with earlier observations on E. coli β-galactosidase and Staphylococcus aureus nuclease substituted with norleucine (5, 6), as well as on α-amylase of Bacillus subtilis with ethionine (29).

When a methionine auxotroph strain of E. coli is grown in the presence of a restricted amount of methionine and an excess of norleucine, a final substitution rate of about 50% is attained. About one further doubling of the bacterial population takes place after methionine has been exhausted. The protein species synthesized during the last bacterial doubling following methionine exhaustion contains almost exclusively norleucyl residues at methionine sites. On the other hand, during the early phase of growth, norleucine does not enter the translation pathway because of the large bias of the Met-tRNA ligase toward methionine (30). In contrast, the methionine permease shows no marked preference for the natural amino acid (31).

After the growth completion of strain PMX in limited methionine and excess norleucine, a population representing about 20% of active adenylate kinase survives to CNBr treatment. Knowing that the overall substitution rate of methionine with norleucine is 50% (compare norleucine and methionine contents in Table 1, column C), even if one (wrongly) assumes that the remaining 30% (i.e. 50–20%) of norleucine residues are randomly scattered within 80% (i.e. 100–20%) of the protein chains, a substantial proportion of at least 12% (i.e. 100 × (1 − (0.5–0.2))6) of the chains should be devoid of norleucine. Therefore, one might envision each protein in the cells of such stationary cultures as an entire distribution of molecular species with defined sequences of methionines and norleucines, both ends of the spectrum, one free of the natural amino acid and the other free of the analog, being grossly over-represented.

The thioether group of methionine makes this amino acid susceptible to oxidizing agents, in particular to hydrogen peroxide. In vivo incorporation of norleucine might thus help overcome the susceptibility inherent to some proteins that is due to the presence of a critical methionyl residue. In order to assess the validity of this notion, we subjected adenylate kinase to hydrogen peroxide treatment under denaturing conditions and showed that its stability toward this reagent increased dramatically when the enzyme contains norleucine instead of methionine. These results are consistent with a purely structural role for the 6 methionine residues of E. coli adenylate kinase. Kres and Noda (32) showed that the modification of 2 to 3 methionyl residues of rabbit muscle adenylate kinase with iodoacetic acid (the thiol groups being previously blocked and later unblocked) was accompanied by complete loss of activity. However, a conclusion was not drawn as to whether derivatization had directly affected the catalytic mechanism or had modified the conformation.

In light of the data presented above, we favor the idea that phenotypic substitution of methionine by norleucine might conveniently overcome the need to engineer this particular residue in oxidation-prone proteins (24, 33). Indeed, the insertion of a single leucine or valine (taken as conservative choices) at one methionine site of a protein can result in a decreased activity (24, 33) whereas the replacement of all methionine residues by norleucine seems to maintain the catalytic properties of enzymes (5, 6). Amino acid analogs have hitherto mainly been used for selecting mutants with enhanced defense mechanisms toward them (34). In the future they will perhaps serve more constructive goals like tuning the activity or improving the stability of certain proteins, without resorting to directed mutagenesis.

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