Mutagenic Analysis of AMP Nucleosidase from *Escherichia coli*

DELETION OF A REGION SIMILAR TO AMP DEAMINASE AND PEPTIDE CHARACTERIZATION BY MASS SPECTROMETRY*

(Received for publication, November 12, 1992)

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AMP nucleosidase (EC 3.2.2.4) from *Escherichia coli* and AMP deaminase (EC 3.5.4.6) from bakers’ yeast are proposed to regulate cellular AMP levels under allosteric control of the activator ATP and the inhibitor, PO₄. Both enzymes contain catalytic sites which bind AMP and regulatory sites which bind ATP. The deduced amino acid sequences of the proteins revealed only one region of homology in which six of eight amino acids are identical. A similar sequence is found in glyceraldehyde-3-phosphate dehydrogenase, *phoE*, ras proteins, RNA polymerase, K⁺-ATPase, nucleolin, and other proteins expected to have nucleotide or phosphate binding properties. In the crystal structure of glyceraldehyde-3-phosphate dehydrogenase, this sequence is part of the NAD⁺-binding site. The function of these amino acids was explored with a deletion mutant of AMP nucleosidase. The protein was over-produced in a pTZ construct using the AMP nucleosidase promoter which resulted in approximately 30% of the total protein as the desired enzyme. The mutation was characterized by DNA sequence analysis and by direct analysis of the peptides using high performance liquid chromatography-mass spectrometry. Deletion of amino acids 128–135, corresponding to DGSELTLD, produced an enzyme with a 20-fold decrease in *V*ₘₐₓ but with smaller changes in substrate saturation kinetics, activation by MgATP, inhibition by inorganic phosphate, and inhibition by the tight-binding inhibitor, formycin 5-phosphate. The deletion mutant of AMP nucleosidase exhibits hysteresis in establishing a steady-state rate of product formation which is most pronounced in the absence of MgATP. These results establish that the sequence DGSELTLD in *E. coli* AMP nucleosidase is not required for binding of AMP, MgATP, or inorganic phosphate. However, the mutant enzyme has a structural defect related to the polymerization state which delays the onset of catalysis and decreases the catalytic efficiency.

Both prokaryotes and eukaryotes contain enzymes which regulate the cellular adenine nucleotide pool by the hydrolytic degradation of AMP. In prokaryotes, degradation occurs through hydrolysis of the N-glycosidic bond to yield adenine and ribose 5-phosphate (Schramm and Leung, 1978a). In eukaryotes, AMP deaminase carries out a similar function by deaminating AMP to IMP and ammonia (Chapman and Atkinson, 1973). No prokaryotes are known to contain AMP deaminase and no eukaryotes are known to contain AMP nucleosidase. Regulatory features of AMP nucleosidase and AMP deaminase are remarkably similar. Both are allosterically activated by ATP, and both are inhibited by inorganic phosphate (Schramm, 1974; Merkler et al., 1989). The hypothesis that AMP nucleosidase evolved into AMP deaminase as prokaryotes evolved into eukaryotes has been tested by cloning and sequencing the genes for these enzymes from *Escherichia coli* and from *Saccharomyces cerevisiae* (Leung et al., 1989; Meyer et al., 1989). Neither of the deduced sequences contained the consensus sequence for adenylate-binding proteins. The deduced amino acid sequences indicate only a small region of homology. Since both AMP nucleosidase and AMP deaminase contain ATP- and AMP-binding sites, the absence of adenylate consensus sequences is surprising and requires verification. The common feature between these proteins is a small region of eight amino acids which is similar to peptides known to be involved in nucleotide or phosphate binding in other proteins including a well characterized domain of glyceraldehyde-3-phosphate dehydrogenase (Rossman et al., 1975).

In this article, the common sequence between AMP deaminase and AMP nucleosidase has been removed by deletion mutagenesis. The mutant protein has been produced and characterized in order to provide information on the nature of the sequence which is common to deaminase, nucleosidase, and other proteins which are known to have nucleotide- or phosphate-binding regions. The results establish that a deletion of eight amino acids changes primarily the catalytic efficiency and the polymeric state of the enzyme. The deduced structure of both the mutant and native AMP nucleosidase is experimentally verified by mass spectral analysis of peptides produced by proteolysis. This procedure ensures that the amino acid composition deduced from DNA sequence reflects the actual amino acid sequence.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases and DNA ligase were purchased from New England Biclabs. Sequenase (T7) DNA polymerase, Sequenase DNA sequencing kit, and pTZ18U were obtained from U.S. Biochemicals Corp. Oligonucleotide-directed in vitro mutagenesis kit and [α-³²P]dATP·8 were acquired from Amersham Corp. Mono Q HR 5/5 fast protein liquid chromatography columns and DEAE-

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cloned into M13mp18 (Leung and Schramm, 1984). The single line the 2.65-kb HinfI fragment restricted initially from pHL8 and sub-
to the native amn gene at the HindIII-PstI sites. The start 
tated amn DNA from the M13 subclone marked in black was ligated 
Sephacel were obtained from Pharmacia LKB Biotechnologies. L-1-
Staphylococcus aureus VS protease,' all protease inhibitors, and lyso-
Tosyl-amido-2-phenylethyl 
the end (1450) of the amn gene are marked. The double lines represent 
Albert Einstein College of Medicine using Applied Biosystems models 
and DNA sequencing were produced by the DNA synthesis facility, 
zyme were from Sigma. Oligonucleotides for the deletion mutagenesis 
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The abbreviations used are: V8 protease, S. aureus 
the gene for E. coli AMP nucleosi-
dase; HPLC, high performance liquid chromatography; MS, mass 
spectrometry; kb, kilobase(s); LC, liquid chromatography.

The modified flowprobe was adapted from a method presented at 
the 2nd International Symposium on Applied Mass Spectrometry in 
the Health Sciences, Barcelona, April 17–20, 1990, by P. L. Jacobs, 
G. J. H. Schmeits, and M. P. de Vries.  

PBZ-Δ(382-405)amn

Fig. 2. DNA sequence of native amn and Δ(382-405)amn in the region of the mutation. Lanes 1–4 represent the sequencing ladder of M13-amn single-stranded DNA template annealed to a sequencing primer (5′-CCA CCA CGA TTA CTC GCC C). Lanes 5–8 show the sequencing ladder of M-13-Δ(382-405)amn single-stranded DNA annealed to the same primer in the region of the deletion. The nucleotides detected from Δ(382-405)amn correspond to the underlined region in ATC GAT GCC TCT GAA TTG ACA CTT GAT CGC.

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Table I

| Protein          | Activity | Specific Activity | Purification | Yield |
|------------------|----------|------------------|--------------|-------|
|                  | mg       | μmol/min         | μmol/mg      | mg   |
| Initial extract  | 5483     | 3078             | 0.56         | 1.0  |
| Heat treatment   | 4182     | 3086             | 0.74         | 1.3  |
| (NH₄)₂SO₄ precipitate | 484     | 831              | 1.7          | 3.0  |
| DEAE-Sephacel    | 120      | 211              | 1.8          | 3.2  |

* Purification was similar to the published method for native en-
zyme (Schramm and Leung, 1978b).

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FIG. 3. Gel electrophoresis of Δ(128–135)AMN samples during purification from HL359 [pT-Z-Δ(382–405)amm]. Protein standards bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonyl anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa) were run in lanes 1 and 8 on a 10% polyacrylamide SDS gel. In lanes 2 and 9 the protein standards, myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), and carbonyl anhydrase (29 kDa) were run. Samples of Δ(128–135)AMN taken during the purification are shown in lanes 3–7: initial bacterial extract (17 μg), heat-treated extract (22 μg), ammonium sulfate precipitate (25–40%) (7 μg), eluate after DEAE-Sephasel chromatography (7 μg), and eluate after MonoQ chromatography (5 μg), respectively. Lanes 10–13 show 1, 5, 10, and 50 μg, respectively, of the MonoQ purified Δ(128–135)AMN. The gels were stained with Coomassie Brilliant Blue and destained with 7% acetic acid.

FIG. 4. Amino acid sequence of AMN showing the 8-residue deletion site of the mutant protein (boxed residues) and peptides observed as ions in the LCMS analysis. Solid lines indicate peptides observed in the analysis of the protein digests. Dashed lines indicate peptides not observed in the analysis of the proteins. All numbers above the peptides are for the combined V₅ and trypsin protease (VT) peptides. The mass range of the analysis was from 450–2200 amu, therefore peptides with 1–3 amino acids are not detected. Peptides from incomplete digestion are indicated, e.g. VT2-VT3, 12–13 etc. Peptide 16m is unique to the mutant protein and excludes the boxed residues.

Sample desorption and ionization was accomplished with an 8-kV neutral Xenon beam from an Ion-Tech saddle-field FAB source. Mass calibrations were performed with cesium iodide and glycerol. Scans were acquired over 15 s from 450 to 2200 atomic mass units using a Finnigan-MAT 90 at a source temperature of 55 °C, a resolution of 1400, and an accelerating voltage of 5 kV.

Initial Rate Kinetic Studies—Initial rates were measured at 30 °C in 1-mL reaction mixtures containing 0.1 M triethanolamine-HCl, pH 8.0, and the indicated concentrations of AMP and ATP. The concentration of MgCl₂ was equal to or greater than the ATP concentration to convert ATP to MgATP and to maintain free MgCl₂ between 10 μM and 1 mM. The reaction was initiated by the addition of 5 μL of enzyme which had been diluted into 0.5 M triethanolamine, pH 8.0. Reactions were terminated by the addition of 0.3 ml of alkaline formycin (Schramm and Hochstein, 1971) or by 20 μM formycin 5-phosphate, an inhibitor of the enzyme (DeWolf et al., 1979).

Assays with Δ(128–135)AMN gave initial rates which lagged for 1 or more min followed by product formation as a linear function of time. Initial rates were calculated from the linear portion of the product formation plots. Three or more time points were used to estimate steady-state reaction rates. Kinetic constants were obtained from fits of the data to the appropriate equations (Cleland, 1977), where appropriate. Preincubation of the enzyme in dilute buffer solutions prior to assay did not eliminate the hysteresis.

Presteady-state Kinetic Studies—Rapid reaction kinetics were conducted in a Kin Tech rapid quenching instrument at 30 °C. All solutions were buffered at pH 8.0 with 0.1 M triethanolamine, pH 8.0. The enzyme syringe contained 20 μM (1.04 mg/ml) native or mutant AMP nucleosidase and was rapidly mixed with an equal volume of substrate AMP nucleosidase and was rapidly mixed with an equal volume containing the indicated concentrations of substrate and allosteric activator. The mixing time of the instrument is approximately 2 ms. Substrate AMP was mixed with 450-2200 amu, therefore peptides with 1–3 amino acids are not detected. Peptides from incomplete digestion are indicated, e.g. VT2-VT3, 12–13 etc. Peptide 16m is unique to the mutant protein and excludes the boxed residues.
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Fig. 5. LCMS chromatograms of the tryptic/V8 digest of AMP nucleosidase native protein. Lower panel, reconstructed total ion current plot. Upper panels, selected ion chromatograms for some of the predicted peptides. Chromatographic conditions: water/glycerol/acetoniitrile/trifluoroacetic acid (92:5:3:0.1 (solvent A)), acetonitrile/water/glycerol/trifluoroacetic acid (50:45:5:0.1 (solvent B)), with a gradient program of 0–100% B in 30 min after an initial isocratic hold of 5 min at 0% B. The flow rate was 10 µl/min.

RESULTS

Preparation of Δ(128–135)AMP Nucleosidase—A deletion mutant protein of AMP nucleosidase [Δ(128–135)AMN] was prepared by oligonucleotide-directed mutagenesis, as described under “Experimental Procedures.” The mutated double-stranded DNA from individual M13 isolates was screened by ClaI restriction analysis. Since the ClaI site in amn was deleted during the mutagenesis the desired mutations gave restriction fragments of 2.9 and 7 kb whereas native amn DNA gave three bands of 1.3, 2.9, and 5.7 kb. One-third of the M13 progeny were shown to have lost the amn ClaI site.

The deoxyribonucleotides 1–500 surrounding the deletion of the mutated Δ(382–405)amn gene were sequenced and compared with the sequence of the parental gene (Leung et al., 1989) (Fig. 1). The results verified that the deletion had occurred in the DNA. No other mutations were observed in deoxyribonucleotides 1–500 of the coding region of the amn gene. A pTZ-Δ(382–405)amn construct was prepared containing the promoter and bases (1–477) of the Δ(382–405)amn gene. The mutant DNA was ligated to the remainder of the native amn gene at the HindIII-PstI sites (Fig. 2). The DNA sequence of bases –24 to 500 of the final construct confirmed the DNA structure. The pTZ-Δ(382–405)amn gene was transformed into HL359, an E. coli strain deficient in native AMN activity (Leung and Schramm, 1984) and devoid of AMN protein as shown by Western blot analysis.

The Δ(128–135)AMN protein was purified using a proce-
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FIG. 6. Selected ion chromatograms of peptides characterizing native and Δ(128–135)AMN. Panels A-D and E-H are selected ion chromatograms for tryptic/V8 digests of native and deletion mutant AMP nucleosidases. The two peptides unique to the native sequence are shown in panels A and D. The peptide VT66, common to both proteins, is shown in panels B and F. The peptide eluting at 62 min at m/z 1021 in panel G is unique to the mutant protein and is not seen in the wild-type protein (C). Spectra obtained by accumulating the scans associated with the chromatographic peak were used to determine their m/z values and allow assignment to the appropriate peptide. The relative intensities in panels A, B, D, F, and G were normalized to the most intense peak signal. The relative intensity of panel C was normalized to that of panel G, while those of panels E and H were normalized to that of A and D, respectively. The retention times of peptides from both digests were within 2% of each other after 60 min of chromatography at 10 μl/min. Equal quantities of peptide digest were applied for analysis of AMN and mutant digests.

The procedure similar to the published method of Schramm and Leung (1978b) (Table I). Denaturing SDS gel electrophoresis of the protein at each step of the purification of Δ(128–135)AMN indicated that the mutant protein was expressed at >30% of total extracted protein and was purified to >99% homogeneity (Fig. 3).

Protease Digestion of Native AMN and Δ(128–135)AMN—Native AMP nucleosidase and mutated Δ(128–135)AMN were treated with V8 protease or trypsin, either separately or in combination. Incomplete protease digestion and/or recovery of peptides from both native and mutant proteins was evidenced by the presence of protein precipitates. Precipitates were removed by centrifugation prior to analysis by mass spectrometry. Trypsin digestion yields two large peptides of Mr, 4867 for AMN and 4036 for Δ(128–135)AMN in the region of the deletion. For FAB mass spectrometric analysis, smaller peptides were generated by combined digestion with trypsin and V8 protease.

Mass Spectral Analysis of Native and Δ(128–135)AMN Peptides—The product of V8 protease/trypsin (VT) digestion was analyzed using HPLC-MS. The spectra and chromatograms of similar analyses were compared for peptides from the wild-type protein and for the mutant. The peptides VT16 and VT17, comprising sequence positions 121–131 and 132–136 were predicted to appear only in digests of native AMN. A single peptide, VT16m, spanning sequence positions 121–128 of the mutant protein, was predicted to be unique to the mutant protein, since it contains residues which span the deletion site (Fig. 4).

Direct mass spectral analysis of the HPLC effluent was used to provide masses of all peptides within the range 450–2200 atomic mass units which were present in the digest.
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**Fig. 7.** Mass spectra of the peptides unique to the native and Δ(128-135)AMN digests. Accumulated scans corresponding to the peaks in Fig. 6 are displayed for AMN (A–C) and the corresponding regions in the chromatogram of the mutant protein (D–F). Peptides VT16 and VT17 are unique to native AMN while VT22 is common to both. Peptide VT16 m is unique to Δ(128-135)AMN. The approximate relative intensity of the signals was 4:1 for panels A and D, 1:16 for panels B and E, and 1:2 for panels C and F.

lower panel of Fig. 5 shows the reconstructed total ion current chromatogram plot of a 50 μg (930 pmol) injection of the cryptic/Vs digest of AMN. The bulk of the peptides eluted in two main clusters, between scans 70-170 and scans 280-380. Selected ion chromatograms for some of the predicted peptides are shown in the panels above the total ion current trace in Fig. 5.

The ions at m/z 617.4 and 1252.6 in Fig. 6, A and D confirmed the presence of both peptides VT17 and VT16 unique to native AMN. Panels E and H show the selected ion chromatograms for corresponding m/z values of the LCMS analysis of the mutant protein. The peptide eluting at 62 min with m/z 1021 ± 1 (panel G) was consistent with VT16m, the unique mutant peptide. Thus, the mutant is missing amino acids 128-135 of the native AMN sequence.

The peptide assignments were confirmed by the examples of mass spectra in Fig. 7, in which the scans corresponding to some of the peaks in Fig. 6 were accumulated and the background was subtracted. The upper spectra of Fig. 7 show that both VT17 (m/z 617.5) and VT16 (m/z 1253.1) are present in the AMN digest, but not in the digest of the mutant protein (lower spectra). Peptide VT22 is expected and is found in both the native and mutant proteins (Fig. 7, B and E). The VT16 and VT17 peptides are replaced in the Δ(128-135) AMN by peptide VT16m (m/z 1021.0) which is clearly detected as a major peak in the mutant but not the native digest (compare Fig. 7, C and F).

Table II summarizes the results of the LCMS analysis of both proteins. The m/z values of the assigned peptides were generally within 0.5 mass units of the predicted values. The highest mass peptide observed was VT42 (m/z 2126.10) and was observed as a weak ion in the digest of the mutant protein. Several ions were assigned to products of incomplete digestion, i.e., peptides which contain a lysine, glutamic acid, or arginine in their internal sequence. The peptides VT59 and VT60 were not expected because it would require the cleavage of a lysine-proline peptide bond. Such bonds are resistant to cleavage by trypsin. The ion at m/z 903 could be attributed to peptides VT26, VT34, or both. Of the peptides which required the cleavage of an Arg-Pro peptide bond, only VT12, VT13, and VT45 were observed, while peptides VT37, VT38, VT40, and VT41 were not observed in either digest.

**Kinetic Properties of Native and Δ(128-135)AMN—Initial rate studies with Δ(128-135)AMN gave a lag period of 1–2 min before a linear rate of product formation was observed when MgATP was present (Fig. 8A). This hysteresis was not observed with the native enzyme (Fig. 8C). In the absence of MgATP, the hysteresis was extended to 20 min or more, making initial rate measurements difficult (Fig. 8B). Preincubation of enzyme in buffer of lower ionic strength, with and without MgCl₂, AMP, formycin 5-phosphate, ATP, and MgATP did not eliminate the hysteresis, suggesting that multiple catalytic turnovers are required to form a stable catalytic unit. In stopped-flow studies, using 10 μM Δ(128-135)AMN or native AMN, no hysteresis was observed in the absence or presence of ATP (Fig. 9). The concentration of Δ(128-135)AMN was 0.02-0.2 μM in kinetic studies. The hysteresis is therefore consistent with a concentration-dependent protein polymerization which is linked to catalytic function. Initial rate measurements for Δ(128-135)AMN were taken after the lag was complete. Because of the relatively long delays under some conditions (Fig. 8), the initial rates are not highly reproducible.

The AMP saturation curve for native AMP nucleosidase in the absence of allosteric activator gave a half-saturation value of 15 mM. The estimated S₅₀ for AMP with Δ(128-135)AMN
Menten kinetics. The value of 0.42 bmol/min/mg. Both enzymes exhibited sigmoidal responses of initial rates to AMP concentration. The substrate to 90 and 210 PM for the native and mutant enzymes. The enzyme had a $K_m$ of 23 mM using steady-state rates established after 30 min.

Addition of MgATP caused a decrease in the values for $V_{max}$ and the $A(128-135)$AMN had a $K_{cat}/K_m$ of 40 and 30 nM for the native and mutant enzymes. These values are in good agreement with those previously reported for AMP nucleosidase and also inhibits $A(128-135)$AMN. The inorganic phosphate. With equivalent fixed concentrations of AMP and MgATP, the native and $A(128-135)$AMN gave apparent inhibition constants of 0.5 mM. The apparent activation constants for MgATP were 22 and 100 $\mu$M for native and $\Delta(128-135)$AMN.

Table II
Summary of peptides in mass range 450-2200 atomic mass units from AMN and AMN mutant protein

| V8/Tryptic peptide | Mass determinations | Sequence position |
|-------------------|---------------------|------------------|
| VT1               | 506.24              | 1-4              |
| VT2               | 1157.62             | 5-16             |
| VT3               | 723.36              | 17-22            |
| VT4               | 772.47              | 23-29            |
| VT5               | 1138.54             | 30-40            |
| VT6               | 473.22              | 41-44            |
| VT12              | 1455.72             | 77-89            |
| VT13              | 633.37              | 90-94            |
| VT14              | 825.28              | 95-99            |
| VT16m             | 1020.59             | VT1020.6 VT121-128 |
| VT16              | 1252.61             | VT121-131        |
| VT17              | 617.36              | VT132-136        |
| VT18              | 822.41              | 137-144          |
| VT19              | 757.34              | 145-150          |
| VT20              | 745.37              | 151-157          |
| VT21              | 1103.50             | 158-167          |
| VT22              | 1176.58             | 168-177          |
| VT24              | 897.44              | 179-185          |
| VT26              | 903.42              | 188-195          |
| VT27              | 1699.85             | 196-208          |
| VT28              | 523.22              | 209-212          |
| VT34              | 903.49              | 249-256          |
| VT37              | 1140.62             | 287-296          |
| VT38              | 1398.64             | 297-308          |
| VT41              | 1501.82             | 313-325          |
| VT42              | 2126.10             | 326-345          |
| VT44              | 781.41              | 349-355          |
| VT45              | 531.33              | 356-360          |
| VT51              | 1064.52             | 371-380          |
| VT54              | 767.41              | 386-392          |
| VT55              | 636.35              | 393-397          |
| VT56              | 748.36              | 398-404          |
| VT57              | 1037.54             | 405-414          |
| VT59              | 1294.67             | 417-428          |
| VT60              | 552.28              | 429-433          |
| VT62              | 755.42              | 436-442          |
| VT63              | 458.19              | 443-445          |
| VT64              | 476.24              | 446-450          |
| VT65              | 836.51              | 451-457          |
| VT66              | 700.44              | 458-463          |
| VT69              | 512.29              | 469-472          |
| VT72              | 510.22              | 476-479          |
| VT73              | 516.29              | 480-483          |

| Products of incomplete digestion |
|----------------------------------|
| VT2-VT3                          | 1861.95             | 5-22              |
| VT4-VT49                         | 659.41              | 364-368           |
| VT2-VT23                         | 1007.51             | 476-483           |
| VT23-VT23                        | 963.65              | 178-185           |
| VT12-VT13                        | 2070.10             | 77-94             |

*Identified as fragment from incomplete digestion.
**Peptide unique to mutant protein.
#Peptide unique to AMN.
$^d$Not predicted (required Lys-Pro cleavage).

**Table II**
Summary of peptides in mass range 450–2200 atomic mass units from AMN and AMN mutant protein

Bold type indicates unique peptides.

Fig. 8. Initial rates of product formation and hysteresis from $\Delta(128–135)$AMN. In panel A, the reaction mixtures contained 0.5 mM ATP, 1 mM MgCl$_2$, and the indicated concentrations of AMP. In panel B, reaction mixtures contained 20 mM AMP and 1 mM MgCl$_2$. For comparison, the initial reaction rates for native AMP nucleosidase are shown in panel C. The squares represent product formation at 2 mM AMP, 0.5 mM ATP, and 1 mM MgCl$_2$ while the diamonds represent product formation at 20 mM AMP and 1 mM MgCl$_2$. In all cases (A–C) reactions contained 0.1 M triethanolamine, pH 8.0, and were initiated by the addition of enzyme.

was 23 mM using steady-state rates established after 30 min at 30 °C (see Fig. 8). Under these conditions, the native enzyme had a $V_{max}$ of 40 and the $\Delta(128–135)$AMN had a $V_{max}$ of 0.42 μmol/min/mg. Both enzymes exhibited sigmoidal responses of initial rates to AMP concentration.

Addition of MgATP caused a decrease in the $S_{0.5}$ values for substrate to 90 and 210 μM for the native and mutant enzymes and caused substrate-saturation to conform to Michaelis-Menten kinetics. The $V_{max}$ value was unchanged for the native enzyme but increased 5-fold for $\Delta(128–135)$AMN in response to MgATP. The kinetic constants for substrate, activator, and inhibitors are summarized in Table III. Activation by MgATP gave a sigmoidal response of initial rate to activator concentration when AMP was fixed at concentrations below 1 mM. The apparent activation constants ($K_{act}$) for MgATP were 22 and 100 μM for native and $\Delta(128–135)$AMN.

Inhibition of AMP nucleosidases occurs with inorganic phosphate. With equivalent fixed concentrations of AMP and MgATP, the native and $\Delta(128–135)$AMN gave apparent inhibition constants of 200–250 μM (Table III). Inhibition occurs in the presence and absence of MgATP, but the observed inhibition constants are greater in the presence of MgATP.

Formycin 5-phosphate is a competitive inhibitor of native AMP nucleosidase and also inhibits $\Delta(128–135)$AMN. The inhibition constants were estimated from titrations of activity as a function of formycin 5-phosphate with fixed concentrations of AMP. Formycin 5-phosphate gave $K_i$ values of 72 and 58 nM for the native and mutant enzymes. These values are in good agreement with those previously reported for AMP
nucleosidases from other sources (DeWolf et al., 1979; Leung and Schramm, 1980).

**Pre-steady State Kinetics of Native and Δ(128–135)AMN**—In contrast to the hysteresis of steady-state measurements (Fig. 8), rapid reaction kinetics at a catalytic site concentration of 10 μM resulted in a linear rate of product formation for the first turnover as well as two subsequent turnovers for both the native and Δ(128–135)AMN (Fig. 9). The reaction rate for native enzyme (Fig. 9A) gives a specific activity of 5.4 μmol/min/mg compared to a $V_{\text{max}}$ of 22 μmol/min/mg in steady-state measurements. The specific activity of the mutant enzyme was 0.09 μmol/min/mg for the first three turnovers compared to a $V_{\text{max}}$ of 2.1 μmol/min/mg in dilute solution following the hysteresic conversion. With both the native and mutant enzymes, the reaction rates are slower than expected based on steady-state kinetics with dilute enzyme solutions. The ratio of activity for the mutant enzyme is 0.09/2.1 = 0.04 as a consequence of enzyme concentration, while for native enzyme the ratio is 5.4/22 = 0.24. Thus, the response of catalytic efficiency to protein concentration differs significantly for the enzymes.

**DISCUSSION**

**Peptide Analysis of Native and Δ(128–135)AMP Nucleosidase**—A majority of the amino acid sequence information deduced from DNA sequencing for AMP nucleosidase has been confirmed by FABMS analysis of protease V8-trypsin digests of the protein. Of the 43 predicted peptides for native and mutant AMP nucleosidases in mass range 450–2,200, direct mass spectroscopic observation was achieved for 31 peptides. These included peptides distributed from amino acid 5 to the C terminus of the 483-amino acid chain. The sequences of peptides 1, 27, 28, 29, 52, and 53 were previously determined by N-terminal Edman sequencing as tryptic peptides. Thus, 37 of the 43 peptides within the observed mass range are confirmed. The peptides which were not detected by FABMS include the cysteine-containing peptides and peptides with masses above 2,200. Di- and most tripeptides were not included in the analysis. Cysteines were not protected in this protocol. No inconsistencies were found between the peptide mass analysis and the DNA sequence, except for a typographical error in the original report (Leung et al., 1989) which converts Val$^{70}$ to Gly$^{70}$.

The structure of the mutated enzyme was confirmed from the DNA sequence and the direct observation of the new peptide VT16m, which results from the elimination of Glu$^{73}$, the site of a V8 protease cleavage. Peptide VT16m replaces VT16 and VT17 of the normal enzyme. All predicted changes were readily observed in the FABMS data.

**Properties of Δ(128–135)AMN**—The deduced amino acid sequences from yeast AMP deaminase and E. coli AMP nucleosidase were compared to test the hypothesis that the deaminase had evolved from the nucleosidase (Meyer et al., 1989). Both of these enzymes contain catalytic sites for AMP, allosteric activator sites for ATP, and allosteric inhibition by PO4, which is competitive for the ATP sites. These enzymes provide the major pathway for AMP degradation in cell-free extracts of E. coli and S. cerevisiae. In the species tested, only prokaryotes contain AMP nucleosidase and only eukaryotes contain AMP deaminase (Merkler et al., 1989), suggesting an evolutionary replacement of AMP nucleosidase by AMP deaminase.

Despite the circumstantial evidence for functional homology, only a single region of eight amino acids showed strong identity. Neither AMP nucleosidase nor AMP deaminase contain the consensus sequences for adenylylate-binding sites. The amino acid sequences 128DGSELTLD in AMP nucleosidase and 344DGKLLTLD in AMP deaminase might be expected to play a functional role with respect to ATP binding, AMP binding, or catalysis or phosphate binding. Similar sequences are found in a family of proteins which contain binding sites for nucleotides, phosphate, or oligonucleotides (Table IV). In glyceraldehyde-3-phosphate dehydrogenase, this sequence forms a well-defined turn which is in contact with both the adenine and ribose rings of NAD$^+$ at the catalytic site (Rossman et al., 1975). In the ras proteins, this sequence leads to the "phosphoryl group" region which makes contact with the magnesium chelated to the γ-phosphoryl of
Deletion Mutant of AMP Nucleosidase

Table III

| Enzyme            | $S_{0.5}$ for AMP | $V_{max}$ | $K_{cat}$ for ATP | $I_{50}$ for PO$_4$ | $K_i$ for FMP |
|-------------------|------------------|-----------|------------------|---------------------|---------------|
| Native            |                  |           |                  |                     |               |
| −ATP              | 15               | 40        |                  |                     |               |
| +ATP              | 0.09 ± 0.04      | 40        | 22 ± 4           | 200                 | 72 ± 7        |
| Δ(128-135)AMN     |                  |           |                  |                     |               |
| −ATP              | 23               | 0.42 ± 0.12 |                  |                     |               |
| +ATP              | 0.21             | 2.1 ± 0.1 | 102              | 250                 | 58            |

*Initial reaction rates were measured in reaction mixtures containing 1.75 mM MgCl$_2$ with or without 1.75 mM ATP in the assay mixture described under "Experimental Procedures."

*The $V_{max}$ constants are from AMP nucleosidase overexpressed in E. coli which has increased specific activity as a result of simpler purification procedures (Leung et al., 1989; Leung and Schramm, 1984).

*The activation constant for MgATP varies as a function of AMP concentration. Activation constants are reported for 0.1 mM AMP for the native enzyme and for 0.5 mM AMP for Δ(128-135)AMN.

*Phosphate is a competitive inhibitor of ATP for the native enzyme, the $I_{50}$ describes the phosphate concentration to double the activation constant for MgATP.

*The $K_i$ values were determined at fixed concentrations of 1 mM AMP, 0.1 mM ATP, 1 mM MgCl$_2$, and 0.2 mM KCl. The $K_i$ values were calculated from the equation for competitive inhibition with the $S_{0.5}$ values and $V_{max}$ values in the table.

*Some of the values for the native enzyme are from Leung and Schramm (1980).

Table IV

Proteins with similarity to amino acids 126-138 of AMP nucleosidase

Amino acids 126-138 of AMP nucleosidase were compared to the NBRF protein data base, release 92, March 1992 using the FASTA comparison algorithm. Amino acids identical with this region of AMP nucleosidase are underlined. Amino acid similarities of ≥0.3 according to the Dayhoff table (Schwartz and Dayhoff, 1979) are indicated by a double dot under the amino acid. AMP nucleosidase is from E. coli, AMP deaminase from bakers' yeast, G-3-P dehydrogenase is from chloroplast, K-ras is human, RNA polymerase is from tomato spotted with virus, Frz E is from Myxococcus xanthus and is homologous to CheA and CheY of S. typhimurium and is also called gliding motility regulatory protein. PhoE is the phosphate-inducible outer membrane protein of E. coli, the K+-ATPase is from brine shrimp, LysA is from Bacillus subtilis, and nucleolin is from humans.

| Protein       | Amino acid sequence | Refs. |
|---------------|---------------------|-------|
| AMP nucleosidase | 126 V I D G S E L T L D R S M | Leung et al., 1989 |
| AMP deaminase  | 332 F R D G K L T L D E V F | Meyer et al., 1989 |
| G-3-dehydrogenase | 95 V I E G T V F V B P D G | Ferri et al., 1990 |
| K-ras          | 45 V I D G E T C L L D I L D | Barbacid, 1987 |
| RNA polymerase | 2527 V I S G E N L K M D R S D | de Haan et al., 1991 |
| Frz E          | 351 V I F N A D I G V D P S N | McCleary and Zusman, 1990 |
| PhoE           | 162 V I D G L N L T L Q Y Q G | van der Ley and Tommassen, 1987 |
| K'-ATPase      | 497 L I D G T E I P L D N H M | Baxter-Lowe et al., 1989 |
| LysA           | 131 V I D E D E I A L D P F C | Yamamoto et al., 1991 |
| Nucleolin      | 631 E I D G N K V T I D W A K | Srivastava et al., 1989 |

bound MgGTP (Barbacid, 1987; Schlichting et al., 1990). Asp$^5$ in the sequence for K-ras in Table IV corresponds to the carboxylate which chelates the magnesium.

The relationship of amino acids 126-138 of AMP nucleosidase from E. coli to sequences known to interact with nucleotides, magnesium chelates, and phosphates in other proteins also suggested that this region may be a substrate or allosteric site. The allosteric activator is MgATP. AMP binds at the catalytic site and inorganic phosphate also binds in competition with MgATP. The inhibition constants for inorganic phosphate of 200 and 250 μM for native and Δ(128-135)AMP nucleosidases clearly indicated that no functional change had occurred at the phosphate-binding site as a result of this mutation. Likewise, the MgATP activation constants of 22 and 100 μM indicated no substantial difference in the ability of the mutant enzyme to be activated by MgATP. The saturation kinetics with AMP for both the mutant and native enzymes indicated that MgATP caused an apparent increase in AMP affinity by approximately two orders of magnitude.

Without ATP, the enzymes require approximately 20 mM AMP to reach half-$V_{max}$. This constant drops to 90-210 μM when ATP is present. The substrate-binding site for AMP interaction is relatively unchanged by the mutation, with $S_{0.5}$ values differing only about 2-fold with or without activation by MgATP. Formycin 5-phosphate is a strong competitive inhibitor of AMP nucleosidases (DeWolf et al., 1979) and of the mutant enzyme. The binding of competitive inhibitors provides dissociation constants which are independent of the catalytic rate constants which influence $K_i$. Some of the values are from Leung and Schramm (1980).

In contrast to the kinetic constants relating to substrate, inhibitor, and effector binding, the catalytic efficiency of AMP nucleosidase.
\( \Delta(128-135) \text{AMN} \) is substantially reduced by the deletion. In the presence of allosteric activator, where the most efficient catalysis occurs, the \( V_{\text{max}} \) is reduced 20-fold. Since this change occurs without substantial changes in the substrate or activator saturation constants, the effect is likely to be an increased energy barrier for the chemical step. This interpretation has been confirmed by a recent observation that the mutant enzyme exhibits intrinsic kinetic isotope effects.\(^3\) The observed isotope effect with [\( ^2\text{H} \)]AMP is 1.066, similar to the value of 1.077 for the acid catalyzed solvolysis of the glycosidic bond of AMP and slightly greater than the value of 1.061 for AMN from \textit{Azotobacter vinelandii} which is known to represent an intrinsic isotope effect (Mentch et al., 1987).

The mutant enzyme recognizes and binds substrates and effectors similar to the normal enzyme, but fails to efficiently carry the bound substrate through the chemical step of bond breaking. Although catalytically inefficient, it is unlikely that the deletion has removed a catalytic site group. Rather, the altered aggregation state evident from hysteresis and slow reaction rates with concentrated enzyme suggests a modification in subunit interface interactions for this hexameric allosteric protein (Giranda et al., 1989).

Despite the catalytic defect in \( \Delta(128-135) \text{AMN} \), the enzyme is produced in large amounts on the pTZ18 expression vector under control of its own promoter (Leung et al., 1989). The protein is soluble and stable. The major difference from the native enzyme is the hysteresis which occurs during the first minutes of catalysis. Hysteretic effects usually indicate a quasi-stable conformation which requires conversion to a more active form for efficient catalysis to occur. For the deletion mutant the inactive form requires several minutes to be converted to the active form by the reagents of the reaction assay mixture. Preincubation experiments with variations in ionic strength, protein concentration, buffers, MgATP, Mg\(^{2+}\), or formycin 5-phosphate did not reveal conditions which prevented the time-delayed onset of catalysis. Evidence that the mutation altered the aggregation status of the protein comes from the presteady state reaction kinetics. At comparable protein concentrations and reaction conditions, approximately 24% of the native enzyme is catalytically active while only about 4% of the mutant is catalytically functional.

\textbf{Conclusions—} Mass spectrometric methods have confirmed the deduced amino acid structure of AMP nucleosidase and a mutant \( \Delta(128-135) \text{AMN} \) from \textit{E. coli}. The \( \Delta(128-135) \text{AMN} \) mutant adopts a resting protein configuration which causes a hysteresis in catalytic activity. Most of the enzyme is in a reversibly inactive form at subunit concentrations above 10 \( \mu \text{M} \). The deleted region influences the catalytic turnover but not the apparent binding constants for substrate, activator, or inhibitor. The results are most consistent with this region being required for proper subunit interactions which occur during the catalytic cycle. Without amino acids 128–135, the chemical step of catalysis becomes the slow step in the enzymatic reaction cycle.

\textbf{REFERENCES}

Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779–837
Baxter-Lowe, L. A., Guo, J. Z., Bergstrom, E. E., and Hokin, L. E. (1989) FEBS Lett. 257, 181–187
Beyer, S. D., Gisvold, T. J., and Hong, G. F. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3963–3965
Cagnoli, R. M., Moore, W. T., and Fan, T. (1987) Rapid Commun. Mass Spectrom. 1, 15–19
Chapman, A. G., and Atkinson, D. E. (1973) \textit{J. Biol. Chem.} 248, 8309–8312
Cleland, W. W. (1977) Adv. Enzymol. 45, 273–387
Devan, Y., DeIlhner, M. D., and Battey, J. F. (1986) \textit{Basic Methods in Molecular Biology}, Elsevier Sci. Publishing Co., New York
de Haan, P., Kormelinis, R., de Oliveira Rosende, R., van Poolwijk, F., Peters, D., and Goddijn, R. (1991) \textit{J. Gen. Virol.} 71, 2207–2216
DeWolf, W. E., Jr., Fullin, F. A., and Schramm, V. L. (1979) \textit{J. Biol. Chem.} 254, 10868–10875
Ferri, G., Stoppini, M., Meloni, M. L., Zapponi, M. C., and Iadarola, P. (1990) \textit{Biochim. Biophys. Acta} 1041, 36–42
Giranda, V. L., Berman, H. M., and Schramm, V. L. (1989) \textit{J. Biol. Chem.} 264, 15674–15680
Kvalnes-Krick, K., and Jorns, M. S. (1986) \textit{Biochemistry} 25, 6061–6069
Leung, H. B., and Schramm, V. L. (1980) \textit{J. Biol. Chem.} 255, 10867–10874
Leung, H. B., and Schramm, V. L. (1984) \textit{J. Biol. Chem.} 259, 6972–6978
Leung, H. B., Kvalnes-Krick, K. L., Meyer, S. L., deRiel, J. K., and Schramm, V. L. (1988) \textit{Biochemistry} 28, 8726–8733
Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) \textit{Molecular Cloning: A Laboratory Manual}, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
McCleary, W. R., and Zusman, D. R. (1990) \textit{Proc. Natl. Acad. Sci. U. S. A.} 87, 5995–5996
Mentch, F., Parkin, D. W., and Schramm, V. L. (1987) \textit{Biochemistry} 26, 921–930
Meyer, S. L., Kvalnes-Krick, K. L., and Schramm, V. L. (1989) \textit{J. Biol. Chem.} 264, 21422–21430
Meyer, S. L., Kvalnes-Krick, K. L., and Schramm, V. L. (1989) \textit{Biochemistry} 28, 8732–8743
Parkin, D. W., Leung, H. B., and Schramm, V. L. (1984) \textit{J. Biol. Chem.} 259, 5898–5902
Rossman, M. G., Liljas, A., Branden, C. I., and Banaszak, Z. (1975) in \textit{The Enzymes}, 3rd Ed. (Boyer, P. D., ed) pp. 269–369
Sanger, F., Nicklen, S., and Coulson, A. R. (1977) \textit{Proc. Natl. Acad. Sci. U. S. A.} 74, 5463–5467
Schlichting, I., Almo, S. C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wali, A. S., Taylor, J., and Schramm, V. L. (1990) \textit{Nature} 345, 309–315
Schramm, V. L. (1974) \textit{J. Biol. Chem.} 249, 1729–1736
Schramm, V. L., and Hochstein, L. I. (1971) \textit{Biochemistry} 10, 3411–3417
Schramm, V. L., and Leung, H. B. (1978a) \textit{Arch. Biochem. Biophys.} 190, 263–271
Schramm, V. L., and Leung, H. B. (1978b) Methods Enzymol. 51, 263–271
Schramm, V. L., and Leung, H. B. (1978c) \textit{Methods Enzymol.} 51, 263–271
Srivastava, M., Fleming, T. J., and Hong, G. F. (1970) in \textit{Atlas of Protein Sequence and Structure} (Dayhoff, M. O., ed) pp. 353–358, National Biomedical Research Foundation, Washington, D. C.
Swartaas, M., Friesing, P. J., Pollard, H. B., and Burns, A. L. (1989) \textit{FEBS Lett.} 250, 99–105
van der Lee, P., and Tommassen, J. (1987) in \textit{Phosphate Metabolism and Cellular Regulation in Microorganisms} (Torriani-Goriini, A., Rothman, F. G., Silver, S., Wright, A., and Yagil, E., eds) pp. 139–163, American Society of Microbiology, Washington, D. C.
Yamanoto, J., Shimizu, M., and Yamane, K. (1991) \textit{Agric. Biol. Chem.} 55, 1615–1626

\(^3\) X. Ma and V. L. Schramm, unpublished observations.