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Substitution of an Alanine Residue for Glycine 146 in TMP Kinase from *Escherichia coli* Is Responsible for Bacterial Hypersensitivity to Bromodeoxyuridine

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The wild-type TMP kinases from *Escherichia coli* and from a strain hypersensitive to 5-bromo-2′-deoxyuridine were characterized comparatively. The mutation at codon 146 causes the substitution of an alanine residue for glycine in the enzyme, which is accompanied by changes in the relative affinities for 5-Br-UMP and TMP compared to those of the wild-type TMP kinase. Plasmids carrying the wild-type *tmk* gene from *Escherichia coli* or *Bacillus subtilis*, but not the defective *tmk* gene, restored the resistance to bromodeoxyuridine of an *E. coli* mutant strain.

Nucleoside monophosphate (NMP) kinases are key catalysts involved in the cellular turnover of nucleotides (1, 10). Considered homologous enzymes with adenylate kinase as the paradigm, bacterial NMP kinases exhibit in fact high variability, both in their structures and their catalytic properties (3, 5, 6, 15). The most striking example is UMP kinase from *Escherichia coli*, a homohexamer subject to complex allosteric regulation (16).

TMP kinase represents approximately 0.01% of soluble proteins in *E. coli*, a percentage 10 to 20 times less than that for adenylate kinase, the most abundant NMP kinase. The *tmk* gene from this bacterium was recently cloned and sequenced (12), thus opening the way for structural and functional analysis of the enzyme, either isolated or within the intact organism.

In this paper we characterize comparatively the wild-type TMP kinase and a modified form recognized as inducing the hypersensitivity of *E. coli* to 5-bromo-2′-deoxyuridine (BUdR) (4). Our objective was to correlate the changes in the structural properties of TMP kinase with altered kinetics and with bacterial hypersensitivity to a nucleoside analog.

Molecular cloning and DNA sequencing of the *tmk* gene from strain TD205 of *E. coli*. A *tmk*-defective mutant (TD205) was isolated after nitrosoguanidine mutagenesis of strain LD0181 of *E. coli*. It had low TMP kinase activity, accompanied by an elevated pool of TMP and hypersensitivity to BUdR, compared to the parent bacterium (4). To identify the site of the mutation, the *tmk* gene from *E. coli* was amplified by PCR with chromosomal DNA from strains NM554 (11) and TD205 (4) as the matrix. The PCR products were inserted in expression vector pET22b (Novagen), giving plasmids pBLT1110 and pBLT1120, respectively (Table 1). The sequences of cloned *tmk* genes were verified by the dideoxynucleotide sequencing method (14). The sequence of the *tmk* gene from strain NM554 was found to be identical to that previously published (12), whereas that of strain TD205 revealed a G→C transversion at codon 146, responsible for the substitution in the protein of an alanine residue for glycine at position 146 (G146A substitution).

Overproduction and molecular characterization of wild-type TMP kinase and of the G146A variant. Plasmids harboring *tmk* genes were introduced into *E. coli* BL21(DE3)/pDIA17. Bacteria were grown at 37°C in 2YT medium (13) supplemented

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli		
NM554	F$^-$ *araD139* Δ(*ara-leu*)*7696 galE15 galK16* Δ(*lac*)*X74 rpsL* (Str^r) *hsdR2*(r_K^- m_K^+) *mcrA mcrB1 recA13*	11
BL21(DE3)	F$^-$ *ompT* [*lon*] *hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	17
TD205	F$^-$ *leuB6 fhuA2 lacY1 glnV44*(AS) *gal-6* λ$^-$ *tmk-1*(Ts) *zce-297*::Tn*10 cpxB11 hisG1*(Fs) *dcd-1 cdd-50 galP63*? *rpsL104 malT1*(λ^r) *xylA7 mtlA2 metB1 deoA91*	4
B. subtilis 168	Wild-type strain used for sequencing of *B. subtilis* genome	7
Plasmids		
pET22b	ColE1 replication vector harboring a cloning/expression region under the control of the T7 promoter; Ap^r	Novagen Inc.
pDIA17	p15A replication vector carrying the *lacI* gene; Cm^r	9
pBLT1110	pET22b derivative carrying *tmk* gene from *E. coli* NM554	This study
pBLT1120	pET22b derivative carrying *tmk* gene from *E. coli* TD205	This study
pHSP236	pET22b derivative carrying *tmk* gene from *B. subtilis* 168	This study

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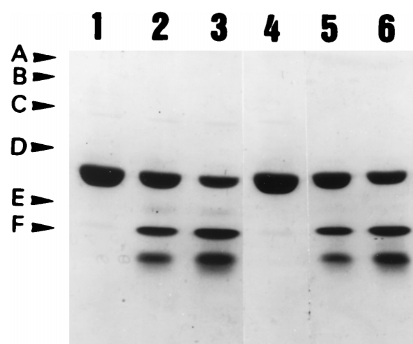

FIG. 1. Proteolysis of *E. coli* TMP kinase by TPCK-trypsin. TMP kinase (wild-type enzyme, lanes 1 to 3; G146A variant, lanes 4 to 6) at 1 mg/ml in 50 mM Tris-HCl (pH 7.4) was incubated at 16°C with TPCK-trypsin (2 μg/ml). At different times (0 min, lanes 1 and 4; 2 min, lane 2; 5 min, lane 5; 6 min, lane 3; 15 min, lane 6) 10-μl aliquots were withdrawn, boiled with electrophoresis buffer, and analyzed by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis and Coomassie blue staining. The arrows indicate the standard proteins (molecular weights are in parentheses): A, phosphorylase *a* (94,000); B, bovine serum albumin (68,000); C, ovalbumin (43,000); D, carbonic anhydrase (30,000); E, soybean trypsin inhibitor (20,100); and F, lysozyme (14,400).

TABLE 2. Kinetic parameters of TMP kinase from *E. coli* with three NMPs[a]

NMP	Wild-type enzyme			G146A variant		
	V_{max}[b]	K_m (mM)	k_{cat}/K_m[c] (s^{-1} M^{-1})	V_{max}	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
TMP	50	0.015	13.2×10^5	11	0.25	17.4×10^3
5-Br-dUMP	40	0.080	2.0×10^5	5	0.28	7.1×10^3
dUMP	30	2.5	4.8×10^3	0.9	4.00	9.0×10^1

[a] Activity was determined at 30°C and 334 nm by coupled spectrophotometric assays using a 0.5-ml final volume and an Eppendorf PCP6121 photometer. The reaction medium contained 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 2 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.2 mM NADH, different concentrations of nucleoside triphosphates (NTPs) and dNMPs, and 2 U each of lactate dehydrogenase, pyruvate kinase, and nucleoside diphosphate kinase. One unit of TMP kinase corresponds to 1 μmol of product formed per min. When NTPs other than ATP were used as the donors, the amount of pyruvate kinase per assay was increased to 20 U.

[b] Units are micromoles per minute per milligram of protein.

[c] k_{cat} was calculated assuming a molecular mass of 23.8 kDa.

with ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml). When the optical density at 600 nm reached 1.5, isopropyl-β-D-thiogalactoside (final concentration, 1 mM) was added to the medium, and the culture was further incubated at 37°C for 3 h. The recombinant enzymes, representing about 30% of total soluble proteins, were purified by Affi-Gel Blue and Ultrogel AcA54 chromatography (2) with the following modification: the enzymes adsorbed onto Affi-Gel Blue were eluted with 2 M NaCl in 50 mM Tris-HCl (pH 7.4). The N-terminal amino acid residues (Met-Arg-Ser-Lys-Tyr-Ile-Val-Ile) determined by Edman sequencing corresponded to those deduced from the *tmk* gene. The molecular masses of TMP kinase measured by electrospray ionization mass spectrometry (23779.6 ± 0.8 Da for the wild-type enzyme and 23794.4 ± 1.4 Da for the G146A mutant) were in agreement with those calculated from the sequences. Gel permeation chromatography and sedimentation equilibrium centrifugation indicated a molecular mass of 48 kDa for each of the two proteins. In other words TMP kinase from *E. coli*, like the enzyme from other sources, is a homodimer. Inactivation of TMP kinase by tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin followed first-order kinetics: $k_1 = 4.1 \times 10^{-3}$ s^{-1} for the wild-type protein and 1.7×10^{-3} s^{-1} for the G146A mutant. Two fragments resistant to further proteolysis were accumulated as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). The sizes of fragments are identical in the two TMP kinases, suggesting the same site of proteolytic attack. N-terminal sequencing of fragments after electroblot transfer indicated that TMP kinase was cleaved in an arginine-rich segment, [147]LKRARARG[154] (Fig. 2). Compared to adenylate kinase and CMP kinase from the same bacterium (3), TMP kinase was less protected against proteolytic inactivation by ATP or other nucleotides. The midpoint denaturation temperatures of TMP kinase determined by differential scanning calorimetry were 57.5°C for the wild-type protein and 60.0°C for the G146A form. Denaturation of TMP kinase by urea is reversible and accompanied by a shift in the maximum of Trp fluorescence, the midpoint transition being at 3.0 M urea for the wild-type enzyme and at 3.3 M for the G146A mutant.

In conclusion, three independent criteria showed that the G146A substitution is accompanied by a small but significant enhancement of the thermodynamic stability of the protein.

Catalytic properties of wild-type and of G146A-modified TMP kinase from *E. coli*. ATP is the best phosphoryl donor of TMP kinase from *E. coli*. The activity with UTP, GTP, and CTP at a single fixed concentration (1 mM) represented 13, 15, and 24%, respectively, of that with ATP. Of the NMPs tested, TMP and 5-Br-UMP are the best acceptors (Table 2). The other halogenated derivatives of dUMP (5-I-UMP and 5-F-

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E.coli          -M-RSKYIVIEGLEGAGKTTARNVVVETLEQLGIRDMVFTREPGGTQLAEKLRSLVLDIKSVGDEVITDKAEVLMFYAARVQ-LVETVI  86
H.influenzae    -M-KGKFIVIEGLEGAGKSSAHQSVVRVLHELGIQDVVFTREPGGTPLAEKLRHL---IKHETEEPVTDKAELLMLYAARIQ-LVENVI  83
B.subtilis      -M-SGLFITFEGPEGAGKTTVLQEIKNILTAEGL-QVMATREPGGIDIAEQIREV---ILNENNILMDPKTEALLYAAARRQHLVEK-V  82
S.cerevisiae    MMGRGKLILIEGLDRTGKTTQCNILYKKLQPNC--KLLKFPER-STRIGGLINE----YLTDDSFQLSDQAIHLLFSANRWE-IVDK-I  80

E.coli          KPALANGTWVIGDRHDLSTQAYQGGGRGIDQHMLATLRDAVLGDFRPDLTLYLDVTPEVGLKR--ARARGELDRIEQESFDFFNRTRAR  173
H.influenzae    KPALMQGKWVVGDRHDMSSQAYQGGGRQLDPHFMLTLKETVLGNFEPDLTIYLDIDPSVGLAR--ARGRGELDRIEQMDLDFFHRTRAR  170
B.subtilis      KPALEQGFIVLCDRFIDSPLAYQGYARGLGIDEVLSINEFAIGDMMPHVTVYFSIDPEEGLKRIYANGSREKNRLDLEKLDFHTKVQEG  171
S.cerevisiae    KKDLLEGKNIVMDRYVYSGVAYSAAKGTNGMDLDWCLQP-DVGLLKPDLTLFLSTQDVDNNAE--KSGFGD-ERYE--TVKFQEKVKQT  163

E.coli          YLELAAQ-----DKSIHTID-ATQPLEAVMDAIRTTVTHWVKE-LDA------  213
H.influenzae    YLELVKD-----NPKAVVIN-AEQSIELVQADIESAVKNWWKS-NEK------  210
B.subtilis      YQELMKRF----PERFHSVD-AGQSKDLVVQDVLKVIDEALKK-IQL------  212
S.cerevisiae    FMKLLDKEIRKGDESITIVDVTNKGIQEVEALIWQIVEPVLSTHIDHDKFSFF  216
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FIG. 2. Alignment of amino acid sequences in four forms of TMP kinase. Strictly conserved residues, expressed in one-letter codes, are outlined, and residues conserved in bacterial TMP kinases are in boldface. Residues supposed to play a role in catalysis are marked by asterisks. The trypsin cleavage site is marked by an arrow, and residues belonging to the LID domain in TMP kinase from *Saccharomyces cerevisiae* are underlined. *H. influenzae*, *Haemophilus influenzae*.

UMP) showed lower V_{max} and higher K_m values for TMP kinase than those exhibited by 5-Br-UMP (data not shown). The K_m for ATP of the wild-type TMP kinase (0.04 mM) was independent of the chemical nature of the cosubstrate. The G146A substitution altered the kinetic parameters of TMP kinase. The V_{max} with ATP and TMP decreased by a factor of 5, and the K_ms for ATP (0.33 mM) and TMP (0.25 mM) increased by factors of 8 and 17, respectively. More significant, however, is the change in the relative k_{cat}/K_m values for 5-Br-UMP and TMP in the two forms of TMP kinase (Table 2). At approximately a twofold excess of 5-Br-UMP over the intracellular pool of TMP in strain TD205, the rate of phosphorylation of 5-Br-UMP will equal that of TMP, whereas for the wild-type strain, a sixfold excess of the analog would have the same effect.

The differences in structure and activity of wild-type and modified TMP kinases from *E. coli* can be rationalized from information obtained for a yeast enzyme (8). Sequence comparisons suggest that Gly146 in the bacterial enzyme is situated in the LID domain (residues 135 to 144 in yeast TMP kinase) (Fig. 2). The LID domain is flexible and mobile, as it is in adenylate kinases, and participates in the conformational change induced by nucleotide binding. It is conceivable that substitution of Ala for Gly146, a residue known to contribute to the flexibility of the polypeptide chain, will restrain the movements of the vicinal amino acids. The increased rigidity of the LID domain might strengthen protein stability but might also reduce the closure of the active site, with a decrease in affinity for ATP and TMP. The variation in the relative affinities for 5-Br-UMP and TMP of the Gly146Ala-modified form versus those of the wild-type enzyme might be rationalized in similar terms. "Distorsion" of the LID domain in TMP kinase resulting from the Gly→Ala substitution affects differently the interactions of the electron-releasing methyl group in TMP and of the electron-withdrawing halogen in 5-Br-dUMP. Therefore, the alteration of the kinetic parameters of TMP kinase resulting from the Gly146→Ala substitution compared to those of the wild-type enzyme might result solely from local structural changes.

Sensitivity to BUdR of strain TD205 transformed with plasmids carrying the *tmk* gene. We investigated the ability of different recombinant plasmids carrying the *tmk* gene from strains NM554 (pBLT1110) and TD205 (pBLT1120) of *E. coli* and from strain 168 of *Bacillus subtilis* (pHSP236) to restore the resistance to BUdR of strain TD205. TD205 and derived strains were grown at 37°C in 2YT medium supplemented with tetracycline (10 μg/ml) and ampicillin (100 μg/ml) when required. When the optical density at 600 nm reached 1, diluted samples containing approximately 100 cells were plated on L broth agar containing tetracycline (10 μg/ml), ampicillin (100 μg/ml), and BUdR (20 μg/ml). The wild-type *tmk* genes from *E. coli* and *B. subtilis* restored the BUdR resistance of strain TD205, whereas the gene encoding the G146A variant did not. As TMP kinase from *B. subtilis* has a specific activity similar to that exhibited by the G146A mutant enzyme (10 U/mg at the optimal substrate concentration) (18), it follows that the BUdR sensitivity of strain TD205 is due to the alteration of TMP kinase substrate specificity and not to a decrease in its specific activity.

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