A T4-phage Deoxycytidylate Deaminase Mutant That No Longer Requires Deoxycytidylate 5′-Triphosphate for Activation*

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A deoxycytidylate (dCMP) deaminase encoded in T4-bacteriophage DNA that is induced on phage infection of Escherichia coli was shown earlier (Maley, G. F., Duceman, B. W., Wang, A. M., Martinez, J. M., and Maley, F. (1990) J. Biol. Chem. 265, 47–51) to be similar in size, properties, and amino acid composition to the T2-phage-induced deaminase. Neither enzyme is active in the absence of dCTP or its natural activator, 5-hydroxymethyl-dCTP. However, on changing the arginine (Arg) at residue 115 of the T4-deaminase to either a glutamate (R115E) or a glutamine (R115Q), the resulting mutant enzymes were active in the absence of dCTP, with each mutant possessing a turnover number or kcat that is about 15% that of the wild-type deaminase. When compared on the basis of specific activity, however, the mutants are about 40–50% of the wild-type (WT)-enzyme’s specific activity. Molecular weight analysis on the wild-type and mutant deaminases using HPLC size exclusion chromatography revealed that the wild-type deaminase was basically a hexamer, particularly in the presence of dCTP, regardless of the extent of dilution. Under similar conditions, R115E remained a dimer, whereas R115Q and F112A varied from hexamers to dimers particularly at concentrations normally present in the assay solution. Activity measurements appear to support the conclusion that the hexameric form of the enzyme is activated by dCTP, while the dimer is not. Another feature emphasizing the difference between the WT and mutant deaminases was observed on their denaturation-renaturation in EDTA, which revealed the mutants to be restored to 50% of their original activities with the WT deaminase only marginally restored.

We and others (1–3) have shown previously that the T2-, T4-, and T6-phage dCMP deaminases (EC 3.5.4.12) require dCTP-Mg2+ in the assay mixture for any enzyme activity to be observed (reviewed in Refs. 4 and 5). The extent of activation was found to be regulated by dTTP, an end product inhibitor of the T4-dCMP deaminase, but in these cases the enzyme is active in the absence of dCTP, but allosterically regulated by the ratio of dCTP to dTTP (4, 5). The cooperative nature of this ligand-protein interaction suggested that the phage and eukaryotic deaminases are oligomeric proteins, a hallmark of allostERIC proteins (6), and each was found subsequently to be composed of six identical Mr 20,000 subunits.

We will demonstrate in the present study that on mutating Arg115 of T4-phage dCMP deaminase to a Glu, the resulting enzyme is highly active in the absence of dCTP-Mg2+ and contrary to the WT-ddeaminase exists as a dimer. A comparison of the protein structure and enzyme activity of R115E with other mutants (R115Q, F112A) and the WT deaminase will also be presented.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—High purity dCMP, dCTP, and dTTP were purchased from Sigma. Other chemicals and reagents used for bacterial cell growth, buffers, enzyme purification and assay, and polyclonal antibodies were obtained from the following commercial sources: Sigma, Fisher, Mallinkrodt Baker, Inc., (Paris, KY), or Life Technologies, Inc. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). DE52 and cellulose phosphocellulose were obtained from Schleicher and Schuell, Inc.

Mutagenesis of T4-dCMP Deaminase—T4-dCMP deaminase mutants were generated using the QuikChange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA) following the manufacturer’s instructions. The plasmid, pET3c-CD5 (7), containing the entire 581-base pair coding region of the wild-type deaminase enzyme was the DNA template used in the QuikChange polymerase chain reaction-based mutagenesis. The codon CGA encoding arginine 115 of the deaminase was changed to GAG (glutamic acid), or CAG (glutamine), to generate the enzyme mutants R115E, or R115Q, respectively. DNA sequence analysis verified that the changed codon sequence at amino acid position 115 in the mutant genes was the only change made in the entire coding region. Wild-type or mutated pET3c-CD5 plasmids were used to transform E. coli BL21(DE3)/pLysS cells (Novagen, Madison WI).

Enzyme Induction and Purification—Wild-type and mutant deaminases were overexpressed from a pET3c-CD5 transformed BL21(DE3)/pLysS host cells as described earlier (7), with the following modifications; bacterial cultures (5 ml) were grown overnight at 37 °C in tryptone-phosphate medium containing 50 µg/ml ampicillin or carbenicillin, and 25 µg/ml chloramphenicol. Stationary phase cultures were added to Fernbach flasks containing 500 ml of the above medium and were shaken (250 rpm) at 30 °C to an Abs600 of 0.6. After adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM the flasks were shaken for another 4–6 h before harvesting the cells by centrifugation. Cell pellets were resuspended in buffer, recentrifuged, and stored at −70 °C. All purification steps, modified from an earlier procedure (8), were carried out at 4 °C unless stated otherwise.

The thawed cell pellet derived from 0.5 liter of induced cells was resuspended in 24 ml of ice-cold TME (10 mM Tris-HCl, pH 8.0, 1.0 mM MgCl2, 0.1 mM EDTA) containing 20 mM 2-mercaptoethanol, 1.0 µg/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride. Resuspended cells were disrupted using a model VC800 Vibra Cell sonicator (Sonics

A similar type of interaction between dCTP and dTTP has been described by us for the eukaryote dCMP deaminases, but in these cases the enzyme is active in the absence of dCTP, but allosterically regulated by the ratio of dCTP to dTTP (4, 5). The cooperative nature of this ligand-protein interaction suggested that the phage and eukaryotic deaminases are oligomeric proteins, a hallmark of allostERIC proteins (6), and each was found subsequently to be composed of six identical Mr, 20,000 subunits.

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which deaminase activity was measured contained 10 mM Tris-HCl, pH 8.5, 10% (w/v) ethylene glycol, 20 mM 2-mercaptoethanol, 0.2 mM MgCl₂, 0.1 mM EDTA) and dialyzed overnight against 1.0-liter changes of Buffer A. The dialyzed samples were incubated at 40 °C. Enzyme purity at this stage was usually 95% as determined by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gels following electrophoresis of the purified protein.

The effect of replacing Arg 115 in WT T4-dCMP deaminase with a glutamine residue was similar in that neither enzyme, unlike the WT deaminase, required dCTP and Mg²⁺ for activity to be observed. Omission of dCTP yielded only marginal activity for the WT enzyme as is evident in the first column in Fig. 1. When Mg²⁺ was omitted too, the enzyme was essentially inactive. In enzyme assays where dCTP was absent from the assay solution, activity was observed immediately upon the addition of WT R115E or R115Q, as shown earlier in Table 1, whereas wild-type enzyme activity could not be detected until dCTP was added to the reaction cuvette (data not shown). In the case of R115Q, it should be noted that while this mutant was about as active as R115E in the absence of dCTP (Table 1), its activity was increased by 30–40% in the presence of 20 μM dCTP, no dCTP was present in assays for the mutant enzymes.

RESULTS

Mutation of Arg¹¹⁵ Results in Loss of Activation by dCTP—The effect of replacing Arg¹¹⁵ in WT T4-dCMP deaminase with either a glutamine (R115Q) or a glutamate (R115E) residue was similar in that neither enzyme, unlike the WT deaminase, required dCTP and Mg²⁺ for activity to be observed. Omission of dCTP yielded only marginal activity for the WT enzyme as is evident in the first column in Fig. 1. When Mg²⁺ was omitted too, the enzyme was essentially inactive. In enzyme assays where dCTP was absent from the assay solution, activity was observed immediately upon the addition of WT R115E or R115Q, as shown earlier in Table 1, whereas wild-type enzyme activity could not be detected until dCTP was added to the reaction cuvette (data not shown). In the case of R115Q, it should be noted that while this mutant was about as active as R115E in the absence of dCTP (Table 1), its activity was increased by 30–40% in the presence of 20 μM dCTP (Fig. 1). When 0.1 mM dTTP was included in the WT deaminase assay the enzyme was inhibited by 20%, with no effect on the mutants. However, in

Adding dCTP (Fig. 1) to reduce its viscosity and then loaded onto a 2.5 × 14-cm DE52 anion exchange column equilibrated with Buffer A. The DE52 column was washed with 3 column volumes of Buffer A (or until the OD₂₈₀ was at background levels), then with 3 column volumes of Buffer B (0.1 M NaCl). Most of the R115E and R115Q deaminase activity eluted in the 0.1 M NaCl wash, while the wild-type deaminase eluted with 0.2 M NaCl. Purification of the deaminase peak was carried out in an Amicon (Amicon Division, W. R. Grace & Co., Beverly, MA) ultrafiltration device (10 kDa cut-off filter), and dialyzed overnight against 1.0-liter changes of Buffer A. The dialyzed samples were incubated at 40 °C. Enzyme purity at this stage was usually 95% as determined by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gels following electrophoresis of the purified protein sample.

Enzyme Activity and Protein Assays—Measurement of enzyme activity and the quantitation of protein in purified deaminase preparations were performed at 30 °C as described earlier (9). The assay solution in which deaminase activity was measured contained 10 mM Tris-HCl, pH 8.0, 0.5 mM dCMP, 0.5 mM MgCl₂, 5 mM 2-mercaptoethanol, with or without 20 μM dCTP, depending on the enzyme to be assayed. One unit of activity refers to the deamination of 1 nmol of dCMP/min under the conditions of the assay. Specific activity refers to units of activity/mg of protein.

Enzyme Kinetic Analysis—dCMP deaminase activity was measured at 5, 10, 50, 100, 250, and 500 μM dCMP in the presence and absence of dCTP. When MgCl₂ was omitted from the assay solution, wild-type enzyme activity was negligible at 0.5 mM dCMP. Substrate Kₘ and Vₘₐₓ values were calculated for the WT dCMP deaminase and the R115Q, and R115E mutants by nonlinear fitting of the kinetic data (catalytic velocities and substrate concentrations) to the Michaelis-Menten equation using the Enzfitter program of Robin Leatherbarrow (version 1.05). Due to the relatively high turnover of the WT deaminase (~2.5 × 10⁴ mol of dCMP deaminated min⁻¹ mol⁻¹ hexamer), enzyme samples were routinely diluted to 15–35 μg/ml protein in a solution of 0.2 M potassium phosphate, pH 7.5, 10% ethylene glycol, 0.1 M 2-mercaptoethanol, and 20 μM dCTP, from which aliquots were removed for the measurement of enzyme activity.

The abbreviations used are: DTT, dithiothreitol; HPLC, high performance liquid chromatography; WT, wild-type.

Fig. 1. Comparative analysis of specific activities of wild-type T4-dCMP deaminase with its mutants, R115E and R115Q. The assay conditions are described under “Experimental Procedures.”

WT
R₁₁₅E
R₁₁₅Q

ADDITIONS

Denaturation-Renaturation Time Course Experiments—One microliter of purified R115E (8.5 μg), R115Q (10 μg), or wild-type enzyme (3.5 μg) was diluted 1:10 in a solution of 0.2 M potassium phosphate, pH 7.5, 6 mM guanidine HCl, 0.1 M 2-mercaptoethanol, ± 1.0 mM EDTA. Control samples were diluted in the absence of denaturant and EDTA. After incubating at room temperature for 4 h, the guanidine concentration was lowered by adding a 49-fold volume of 10 mM potassium phosphate, pH 7.5, 0.1 M 2-mercaptoethanol, ± 1.0 mM EDTA (no EDTA was added to control samples) to allow enzyme renaturation. At 0-, 10-, 20-, 30-, and 40 min intervals after renaturation, 10-μl aliquots of enzyme were assayed for dCMP deaminase activity. Time point assays of WT enzyme were conducted in the presence of 20 μM dCTP; no dCTP was present in assays for the mutant enzymes.

Molecular Weight Determinations—Enzyme samples were analyzed at room temperature using a Waters Protein-Pak 300SW column (8 × 300 mm) integrated into a Waters HPLC system (Waters Corp., Milford, MA) consisting of a model 625 LC, a model 996 Photodiode Array Detector, and a model 717 plus Autosampler. The data were collected and processed (i.e. peak retention time, height, area, etc.) using the Millennium version 2.00 software provided with the HPLC system. Enzyme samples were eluted isocratically in 50 mM potassium phosphate, pH 7.5, 0.2 M NaCl, 0.5 mM MgCl₂, 2.0 mM DTT + 20 μM dCTP at either 0.4 ml/min or 0.8 ml/min. Protein elution was detected at both 280 and 229 nm. The injection loop limited the sample volume to 200 μl (maximum). Mₚ values of WT deaminase, R115E, R115Q, and F112A were obtained by injecting 50 μg of each protein in 20 μl onto the column. Molecular weight standard proteins (Sigma) used to calibrate the column were: α-amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), carbonic anhydrase (29,000), and horse heart cytochrome c (12,400).

RESULTS

Mutation of Arg¹¹⁵ Results in Loss of Activation by dCTP—The effect of replacing Arg¹¹⁵ in WT T4-dCMP deaminase with either a glutamine (R115Q) or a glutamate (R115E) residue was similar in that neither enzyme, unlike the WT deaminase, required dCTP and Mg²⁺ for activity to be observed. Omission of dCTP yielded only marginal activity for the WT enzyme as is evident in the first column in Fig. 1. When Mg²⁺ was omitted too, the enzyme was essentially inactive. In enzyme assays where dCTP was absent from the assay solution, activity was observed immediately upon the addition of WT R115E or R115Q, as shown earlier in Table 1, whereas wild-type enzyme activity could not be detected until dCTP was added to the reaction cuvette (data not shown). In the case of R115Q, it should be noted that while this mutant was about as active as R115E in the absence of dCTP (Table 1), its activity was increased by 30–40% in the presence of 20 μM dCTP (Fig. 1). When 0.1 mM dTTP was included in the WT deaminase assay the enzyme was inhibited by 20%, with no effect on the mutants. However, in
the presence of 0.3 mM dTTP the WT deaminase was inhibited by about 90%, while the mutants were inhibited by about 30% (Fig. 1). Thus, although some inhibition of the mutants can be effected by dTTP, they appear to be much less sensitive to inhibition by dTTP than the WT deaminase, consistent with their lesser sensitivity to dCTP.

**Kinetic Characterization of the Deaminase Mutants**—To determine whether the kinetic properties of the mutants differed, $V_{\text{max}}$ and $K_m$ values of each of the enzymes were evaluated in the presence and absence of dCTP using a nonlinear fitting of the kinetic data to the Michaelis-Menten equation. Table I reveals that the maximal activity ($V_{\text{max}}$) of R115Q and R115E was about 45–55% that of the WT deaminase when measured in the absence of dCTP. At the concentration of dCTP normally employed for the WT deaminase assay (20 M) the activity of R115E was perhaps slightly inhibited, while that of R115Q was increased by 30–40% (data not shown). A similar type of comparative analysis of enzyme activity with the WT deaminase in the presence and absence of dCTP could not be conducted due to the fact this enzyme is basically inactive in the absence of dCTP. The higher $V_{\text{max}}$ (about 2-fold) of the WT enzyme relative to R115Q and R115E could be due in part to its 4–5-fold lower $K_m$, relative to that of the mutants. When the mutants and WT deaminase are compared on the basis of $k_{\text{cat}}$, the differences become even more exaggerated (Table I), since $M_r$, values become a factor in this parameter, and as indicated below the WT enzyme is most likely a hexamer, while the mutants appear to be in their dimeric state during the course of the assay. The differences are even more exaggerated when their specificity constants ([$k_{\text{cat}}/K_m$]) are compared (22 s$^{-1}$ M$^{-1}$ for the WT deaminase versus 0.60 for the mutants).

**Molecular Weight Analysis of T4-phage Deaminase and Mutants**—Earlier studies with the chick embryo (4) and T2-phage deaminases (11) indicated that under conditions of dilution or inhibition by dTTP the enzyme was reduced in size. With the WT and mutant deaminases available to us now, and the improvement in $M_r$ determinations via size exclusion HPLC, a more careful evaluation of the apparent $M_r$ values of these proteins was undertaken using the protocol described under “Experimental Procedures.” A plot of the elution times of the WT T4-deaminase, R115E, R115Q, and F112A, versus log $M_r$ using the standards indicated under “Experimental Procedures” yielded the following respective $M_r$ values, 127,000, 124,500, and 111,900 (data not shown). It should be indicated that 20 mM dCTP was included in the elution buffer.

**Table I**

| Enzyme               | $K_m$ (M) | $V_{\text{max}}$ (pmol/min/mg) | $k_{\text{cat}}$ (s$^{-1}$) |
|----------------------|-----------|--------------------------------|----------------------------|
| WT deaminase         | 21 ± 1.8  | 220 ± 2.0                      | 466                        |
| R115Q$^c$            | 127 ± 3.4 | 121 ± 1.2                      | 85                         |
| R115E$^d$            | 137 ± 11.4| 98 ± 3.1                       | 69                         |

$^a$ $K_m$ and $V_{\text{max}}$ were obtained from nonlinear fitting of data to the Michaelis-Menten equation. The standard deviations were based on three determinations in each case.

$^b$ This value refers to pmol of dCMP deaminated per min/mg of protein at 30 °C, which can also be defined as specific activity.

$^c$ WT deaminase showed no activity in the absence of dCTP + Mg$^{2+}$, but in the presence of Mg$^{2+}$ alone a small amount of activity was noted.

$^d$ Activity was determined in the absence of dCTP.

(22 s$^{-1}$ M$^{-1}$ for the WT deaminase versus 0.60 for the mutants).

Altering the Activation of T4-phage dCMP Deaminase

**Table II**

| Protein concentration | WT          | R115E      | R115Q      | F112A  |
|-----------------------|-------------|------------|------------|--------|
| mg/ml                 |             |            |            |        |
| 4.0                   | 119,000     | 44,500     | 59,100     | 113,000|
| 0.5                   | 122,000     | 43,300     | 50,100     | 105,000|
| 0.001                 | 112,000     | 41,600     | 39,900     | 38,200 |

$^a$ 20 μm in assay solution.

$^b$ 80/20 mixture of hexamer/dimer.

$^c$ 70/30 mixture of hexamer/dimer.

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It is of interest to note that R115Q, like the WT deaminase, was markedly activated by dCTP when its hexameric form was assayed following isolation from the sizing column. The extent of activation was at least five times the activity of the enzyme in the absence of dCTP. By contrast the column-isolated dimer, like R115E, showed little if any activation by dCTP. A similar result was obtained with the M$_r$ 60,000 form of R115Q that was isolated in the absence of dCTP. However, as indicated in Fig. 1, R115Q when assayed directly showed only a 30–40% activation by dCTP, which suggests that when the two forms of R115Q are not separated prior to assay, the dimer predominates.

**Enzyme Denaturation-Renaturation and Zinc Chelation Experiments**—Previous work (9) in our laboratory demonstrated that when T4-phage-dCMP deaminase was denatured in the presence of 6 M guanidine HCl, almost complete restoration of activity could be obtained on removal of the denaturant by dilution or dialysis. Similar studies were conducted with the
R115E and R115Q mutants and comparable with the earlier WT-deaminase denaturation-renaturation results, their respective activities were restored with time (Fig. 3A). It is of interest to note that the WT-deaminase, when refolded, was even more active than prior to the denaturation-renaturation process, which indicates that the renatured enzyme’s structure is more supportive of enzyme activity than that before denaturation. However, when the denaturation of WT-enzyme was conducted in the presence of EDTA to remove its two resident Zn\(^{2+}\) atoms/subunit (9), little activity could be restored on denaturant removal (Fig. 3B), even when Zn\(^{2+}\) was added to the renaturation solution. By contrast, when similar studies were conducted with R115E and R115Q, 54 and 60% of their respective original activities were detected 40 min after renaturation (Fig. 3B).

**DISCUSSION**

Since our initial observation that dCMP deaminase, at least in eukaryotes, is an allosteric enzyme activated by dCTP and inhibited by dTTP (13), a major objective has been to determine how these effectors manage to regulate this enzyme. The T4-(and also T2- and T6-) phage dCMP deaminase may not be considered a classical example of an allosteric enzyme (6), since it does not show kinetic cooperativity, or activity for that matter, in the presence of substrate alone. However, in the presence of dCTP and Mg\(^{2+}\) the enzyme assumes an active conformation and demonstrates a heterotrophic interaction between dCTP and dTTP that is sigmoidal in nature (8). Thus, the phage deaminase could be considered a unique example of an allosteric enzyme, one that is related to its eukaryotic counterpart, but differing somewhat in the way its six subunits interact in the presence of ligands, relative to the manner in which the six subunits of the chick embryo (4) or human deaminase interact (14). The photofixation of dTTP to the T2-deaminase (15), a process inhibited by dCTP, suggests that the regulators probably bind to the same site or to sites that are in proximity to one another. Subsequent studies revealed that dTTP photofixes to Phe\(^{112}\) in the T4-phage enzyme and that mutating this residue to an Ala results not only in the loss of photofixation, but in a partial loss in the enzyme’s dCTP requirement for activity (10). In contrast to the WT-deaminase, F112A is active in the absence of dCTP, but it possesses only about 10% as
much activity. The F112A activity is increased greatly by the presence of 100–300 μM dCTP, reflecting this mutants greatly reduced binding of dCTP (10). The fact that dCTP, in addition to dTTP binding, is reduced considerably suggests a common binding site for these nucleotides. McIntosh and Haynes (16) also implicated this site in the binding of dCTP to several proteins, including dCMP deaminase and ribonucleotide reductase. Thus, the conservation of an Ala-Ala-Arg site in those regulated proteins suggested that the positively charged guanidinium group of Arg might be involved in the binding of dCTP and dTTP through an electrostatic interaction with their negatively charged phosphate groups. In support of this thesis it is seen in this paper that on replacing Arg115 of the T4-phage dCMP deaminase with a Glu, a mutant is obtained that no longer depends on dCTP for activity and that it is much less sensitive to inhibition by dTTP. In this instance as much as 40% of the activity of the R115E mutant was retained (based on specific activity) relative to the WT-deaminase, with little or no response on addition of dCTP to the assay (Fig. 1). The lack of responsiveness of R115E to dCTP contrasts with R115Q, which although as active as R115E, can still be activated by 30 to 40% by the presence of 20 μM dCTP (Fig. 1).

It is perhaps deceptive to compare the activities of the various deaminases on the basis of specific activities alone because of the difference in their molecular weights. Thus, when size is factored in as a result of employing kcat values, that of the WT T4-deaminase is 467 s\(^{-1}\), while in the case of R115E a value of 69 s\(^{-1}\) is obtained. This 7-fold difference contrasts with their specific activities where only a 2-fold difference (Table I) is encountered. In the case of R115E it is clear that this protein is a dimer, but for R115Q its oligomeric state is less certain as it can be a dimer or hexamer or something in between (Fig. 2, Table II). As a dimer the kcat of R115Q is 86 s\(^{-1}\) (Table I), but as a hexamer its kcat would be 255 s\(^{-1}\). It is therefore important to know what the structural status of a protein is in calculating kcat. At the protein concentration of 0.05–1.0 μg employed in the assay of each of the mutant enzymes, all would appear to be dimers (Table II) whether dCTP is present or not. It is not entirely clear that the size of the holoenzyme is affected by the charge of the amino acid residue at 115 but is suggested by the fact that R115Q has less of a tendency to dissociate at protein concentrations comparable with R115E (Fig. 3, Table II), and the presence of dCTP appears to stabilize the hexameric state of this protein. However, when diluted sufficiently R115Q will dissociate to a dimer whether dCTP is present or not (Table II). By contrast the WT-deaminase retains its hexameric state at the highest levels of dilution (1 μg/ml), particularly in the presence of dCTP.

There appears to be a complex relationship between Phe112 and Arg115 in their interaction with dCTP and dTTP, as evidenced by the fact that the mutation of either amino acid affects the binding of these nucleotides. As shown earlier (10) conversion of Phe112 to an Ala greatly reduces the binding of dCTP and dTTP and impairs the photoxidation of the latter. The importance of Phe in this process is emphasized by the fact the human dCMP deaminase, which contains a Thr-Ala-Ala-Arg sequence (17) in a sequence comparable with that of the phage deaminase, does not photoxify dTTP (10). Replacement of Phe112 in the phage enzyme with a Thr, as occurs in the case of the human deaminase, might explain why the human enzyme (14) is not regulated as tightly by dCTP and dTTP. Both residues would thus appear to be important in nucleotide binding, Phe possibly through van der Waals interactions with the pyrimidine ring and Arg via electrostatic interaction with the phosphate of the nucleotides. Whether this proposal is realistic or not might be established through x-ray crystallographic analysis, if reasonable crystals of the WT-deaminase or R115E can be obtained. Meanwhile it would be of interest to determine whether converting the Thr in the human deaminase to a Phe enables this enzyme to now photoxify dTTP, or on converting the Arg corresponding to Arg115 to a Glu promotes dimer formation in the human enzyme, as it does with phage deaminase.

Another difference between the human and phage deaminases is in the number of Zn2+ atoms associated with each subunit. While it was anticipated that Zn2+ would be associated with the active sites of the human and phage deaminases, based on their similarity to other aminohydrolases such as adenosine (18) and cytidine deaminase (19), the presence of a second Zn2+ in each subunit of the phage deaminase was unexpected (9). As indicated previously this Zn2+ may be involved in promoting protein-protein interactions between T4-phage dCMP deaminase and T4-phage thymidylate synthase (20). The role of this second Zn2+ site, as well as its location, is still to be clarified and may provide an explanation for why R115E and R115Q retain at least 50% of their activity on denaturation-renaturation in the presence of EDTA, while the WT-deaminase is at least five times more sensitive to this treatment (Fig. 3B). It is possible that the zinc ions at the active and secondary sites of the WT-deaminase are more exposed to EDTA during denaturation than R115E and R115Q, although preliminary data obtained from sizing analysis has shown that the hexameric state of the WT-deaminase is not restored following denaturation-renaturation in the presence of EDTA, while it is in the absence of EDTA.

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REFERENCES
1. Fleming, W. H., and Bessman, M. J. (1967) J. Biol. Chem. 242, 363–371
2. Soccio, J. J., Panny, S. R., and Bessman, M. J. (1989) J. Biol. Chem. 244, 3608–3706
3. Maley, F., and Maley, G. F. (1972) in Current Topics in Cellular Regulation (Horecker, B. L., and Stadtman, E. R., eds) Vol. 5, pp. 177–228, Academic Press, New York
4. Maley, F., and Maley, G. F. (1970) Adv. Enzyme Regul. 8, 55–71
5. Cohen, S. S. (1968) Virus Induced Enzymes, pp. 89–147, Columbia University Press, New York
6. Monod, J., Wyman, J., and Changeux, J.-P. (1965) J. Mol. Biol. 12, 88–118
7. Moore, J. T., Uppal, A., Maley, F., and Maley, G. F. (1993) Protein Expression Purif. 4, 160–163
8. Maley, G. F., Guarino, D. V., and Maley, F. (1972) J. Biol. Chem. 247, 931–939
9. Moore, J. T., Silversmith, R. E., Maley, G. F., and Maley, F. (1993) J. Biol. Chem. 268, 2288–2291
10. Moore, J. T., Ciesla, J. M., Changchien, L.-M., Maley, G. F., and Maley, F. (1994) Biochemistry 33, 2104–2112
11. Maley, G. F., MacColl, R., and Maley, F. (1972) J. Biol. Chem. 247, 940–945
12. Maley, G. F., Duceman, B. W., Wang, A.-M., Martinez, J., and Maley, F. (1990) J. Biol. Chem. 265, 47–53
13. Maley, G. F., and Maley, F. (1962) J. Biol. Chem. 237, 3311–3312
14. Maley, G. F., Lobo, A. P., and Maley, F. (1993) Biochim. Biophys. Acta 1162, 161–170
15. Maley, F., and Maley, G. F. (1982) J. Biol. Chem. 257, 11876–11878
16. McIntosh, E. M., and Haynes, R. H. (1988) Mol. Cell. Biol. 8, 1187–1202
17. Weiner, K. B., Weiner, R. S., Maley, F., and Maley, G. (1993) J. Biol. Chem. 268, 12633–12639
18. Wilson, D. K., Rudolph, F. B., and Quiocho, F. A. (1991) Science 252, 1278–1284
19. Betts, L., Xiang, S., Short, S. A., Wolfenden, R., and Carter, C. W., Jr. (1994) J. Biol. Chem. 271, 2393–23942
20. McGaughey, K. M., Wheeler, L. J., Moore, J. T., Maley, G. F., Maley, F., and Matthews, C. K. (1996) J. Biol. Chem. 271, 23037–23042
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