Crystal Structures of Vertebrate Dihydropyrimidinase and Complexes from Tetraodon nigroviridis with Lysine Carbamylation

**METAL AND STRUCTURAL REQUIREMENTS FOR POST-TRANSLATIONAL MODIFICATION AND FUNCTION**

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**Background:** Lysine carbamylation facilitates metal coordination for enzymatic activities.

**Results:** Structures of dihydropyrimidinase as the apo- and holoenzyme with one and two metals and its substrate/product complexes are determined.

**Conclusion:** The structures reveal four steps in the assembly of the holoprotein with the carbamylated lysine and two metal ions.

**Significance:** The results illustrate how proteins exploit lysines and metals to accomplish lysine carbamylation and enzymatic functions.

Lysine carbamylation, a post-translational modification, facilitates metal coordination for specific enzymatic activities. We have determined structures of the vertebrate dihydropyrimidinase from Tetraodon nigroviridis (TnDhp) in various states: the apoenzyme as well as two forms of the holoenzyme with one and two metals at the catalytic site. The essential active-site structural requirements have been identified for the possible existence of four metal-mediated stages of lysine carbamylation. Only one metal is sufficient for stabilizing lysine carbamylation; however, the post-translational lysine carbamylation facilitates additional metal coordination for the regulation of specific enzymatic activities through controlling the conformations of two dynamic loops, Ala°–Arg° and Met°Met°, located in the tunnel for the substrate entrance. The substrate/product tunnel is in the “open form” in the apo-TnDhp, in the “intermediate state” in the monometal TnDhp, and in the “closed form” in the dimetal TnDhp structure, respectively. Structural comparison also suggests that the C-terminal tail plays a role in the enzymatic function through interactions with the Ala°–Arg° dynamic loop. In addition, the structures of the dimetal TnDhp in complexes with hydantoin, N-carbamyl-β-alanine, and N-carbamyl-β-amino isobutyrate as well as apo-TnDhp in complex with a product analog, N-(2-acetamido)-iminodiacetic acid, have been determined. These structural results illustrate how a protein exploits unique lysines and the metal distribution to accomplish lysine carbamylation as well as subsequent enzymatic functions.

Post-translational modification of proteins increases the diversity in the proteome (1–3). Carbamylation on lysine extends the residue length by ~2 Å and changes the side chain from a positive to negative charge at neutral pH. This unique modification enables the carbamylated lysine to play multifunctional roles in proteins, especially critical for many physiologically important enzymes to exhibit the proper activities (4, 5). In humans, the proteins involved with lysine carbamylation are found to be related to the human diseases, such as type 2 diabetes, developmental delay, metabolic acidosis, mental retardation, hypotonia, and seizures (6–9). In plants, to convert the atmospheric carbon dioxide to energy-rich molecules, the enzyme Rubisco catalyzes the CO₂ fixation in photosynthesis (10). Rubisco has no enzymatic function until the key lysine residue is carbamylated. The roles of the carbamylated lysine...
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are for metal binding, such as the magnesium ion, as well as to serve as a base for proton abstraction in the catalytic cycle. To counter antibiotics, bacteria have developed the β-lactamase resistance system to destroy the β-lactam antibiotics for survival (11–13). The function of the carbamylated lysine residue in class D β-lactamases is not only to promote the acylation step of the active-site serine but also to activate hydrolytic water for the second catalytic step of the deacylation event (13). So far, there are about 250 structures containing carbamylated lysine residues found in the Protein Data Bank. Although carbamylated lysines exist in a broad range of organisms and have been considered as a residue of catalytic capabilities in nature among the 20 canonical amino acids (14), much less is known about the carbamylation, biochemically and structurally, on the ε-amino group of lysine.

Lysine carbamylation does not need an additional enzyme to achieve the covalent modification, a process that has been reported to be a spontaneous and reversible (15, 16). By comparison, additional specific enzymes or catalysts, such as methyltransferases and aminotransferases, are required to catalyze methylation and acylation reactions, respectively (17, 18), both with high kinetic barriers for the covalent bond formation. Thus, the spontaneous and reversible features of lysine carbamylation are unique in post-translational modifications. In fact, lysine carbamylation is difficult to establish. Most of the carbamylated lysines in proteins are identified by x-ray crystal structures.

In addition to the catalytic role, lysine carbamylation is also involved in the coordination with metal ions. For example, metal ions are found in the amidohydrolase superfamily (19), such as dihydroxypyrimidinase (DHP; EC 3.5.5.2) in animals. The physiological function of DHP is to hydrolyze dihydouracil (DHU) or dihydrothymine (DHT) to the 20 canonical amino acids (14), much less is known about the carbamylation, biochemically and structurally, on the ε-amino group of lysine.

Molecular Cloning and Protein Purification—Healthy green spot pufferfish, T. nigroviridis, were sacrificed. Total RNA was isolated from fish liver using the RNeasy® Protect minikit (Qiagen) according to the manufacturer’s protocol. The RNA was transcribed into the single-stranded cDNA using the SuperScript III first-strand synthesis system for RT-PCR kit (Invitrogen). The cDNA of the recombinant TnDhp was amplified by PCR using the sense primer 5’-GCCGCGATCCATGGGCGAAGCGCAGAGGAGG-3’ and the antisense primer 5’-GCCGCGACGGTTCAGGTCCGAGCAGCAAC-3’ (with the BamHI and EcoRI cutting sites underlined). After digestion by restriction enzymes, a fragment with BamHI and EcoRI sticky ends was purified from agarose gel using the Gene-Spin™ 1-4-3 DNA Purification Kit-V 2.0 (PROTECH) and subcloned into the BamHI/EcoRI precursor plasmid for T 4 DNA ligase. pET 44a (+) harboring the TnDhp cDNA was subsequently transformed into Escherichia coli BL21 (DE3) cells for recombinant protein expression and purification. The bacteria were grown in Luria-Bertani (LB) medium, and protein expression was induced with isopropyl-β-D-thiogalactopyranoside at 25 °C for 30 h. To improve the solubility of the recombinant TnDhp, the fusion protein with native E. coli NusA (Nus-tag) attached to the C terminus (28) was first prepared and then purified and eluted with an imidazole gradient through an Ni 2+ column. After thrombin cleavage of the Nus-tag, the pure recombinant TnDhp (non-amended enzyme) was eluted again through the Ni 2+ column.

Preparation of the Apo- and Metal-amended TnDhps—The apoenzyme was prepared by dialyzing non-amended TnDhp against the chelating buffer (2.5 liters) containing MES (50 mM, pH 6.5), EDTA (1 mM), and 8-hydroxy-5-quinolinesulfonic acid (15 mM) at 25 °C for 4 days with one change of the fresh chelating buffer (2.5 liters). The apo-TnDhp was then desalted through a desalting column (HiTrap; 1.6 × 2.5 cm) with HEPES (50 mM, pH 7.0). The metal-amended TnDhp was prepared by adding zinc chloride (1 mM) or cobalt chloride (1 mM) into the LB medium during isopropyl-β-D-thiogalactopyranoside induction to produce the zinc-amended (mono-Zn TnDhp) or the cobalt-amended TnDhp (mono-Co TnDhp), respectively.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Assays—The metal ion compositions of the various TnDhps were determined by ICP-MS. The Centricon (molecular weight cut-off 30,000; Amicon) was used to replace the buffer solution of each sample with potassium phosphate buffer (10 mM, pH 7.0), which served as the background solution for the determination of the metal ions. The measurement on each sample was independently repeated three times with calculated S.D. values.

Activity Assays—The specific activity measurements were performed by a rapid spectrophotometric assay (27). The TnDhps activity was determined by measuring the decrease of the absorbance at 298 nm upon the hydrolysis of phthalimidase as the substrate at 25 °C. For routine assays, the reaction mixture (1 ml)
TABLE 1

| Cell cultures          | Mol of metal/mol of monomer | Relative activity |
|------------------------|-----------------------------|------------------|
|                        | Calb | Cobalt | Zinc |             |
| Apo-TnDhp              | 0.00 | 0.00   | 0.00 | 0.00         |
| Zinc-amended           | 0.01 | 0.01   | 0.01 | 0.01         |
| Non-amended            | 0.00 | 0.00   | 0.00 | 0.00         |

The molar ratios of metal to protein were determined by ICP-MS.

Metal ions at 1 mM concentration were amended in the cell cultures.

The apo-TnDhp was prepared as described under "Experimental Procedures."
incorporation of zinc ions into the apo-TnDhp could restore the enzymatic activity (Fig. 1). However, the activity curve also shows that concentrations of zinc ions significantly higher than the 1:1 stoichiometry caused a slight inhibitory effect on the protein activity. At a concentration of 25 μM, the activity decreased to 60% of the maximum value (data not shown).

To verify that the binding of zinc ions is specific, we titrated samples at various metal/protein ratios and determined the zinc contents by ICP-MS after the solutions were passed through the desalting column to remove zinc ions that were non-specifically bound (Fig. 1). The ICP-MS data show that all samples contain only one zinc ion per protein monomer, confirming that the maximum activity was obtained with only one metal ion occupying a specific site in the protein.

**Overall Structure and Molecular Packing of TnDhp**—The crystal structures of TnDhp in various states were determined and summarized in Table 2. The structure of apo-TnDhp was first determined. It exhibits a monomer per asymmetric unit in crystals of the space group I4122, with 492 amino acids from Ala4 to Ala495 out of a total of 500 residues (Fig. 2, A and B). The molecular dimensions of TnDhp are about 70 × 55 × 50 Å3, with a surface area of ~18,912 Å² accessible to the solvent. The overall structure comprises 18 α-helices and 20 β-strands and folds into one minor (Ala4–Gly58, Phe384–Lys405, and Pro431–Gly437) and one major domain (Gly59–Asn383 and Thr406–His130). After the last α-helix (Glu466–Glu577), the C-terminal fragment (Val578–Ala595) forms a long tail of 18 amino acids. As is commonly found in DHPs, the major domain folds as a distorted (α/β)8 TIM barrel, whereas the minor domain forms a sandwich-like structure with 10 mixed anti-parallel/parallel β-strands. The electrostatic surface reveals a cleft of about 10 × 10 × 20 Å3 in the center of the major domain that facilitates the entry of substrates to the active site.

**The TnDhp exhibits a monomer in the asymmetric unit of the crystals; however, the crystallographic symmetry-related dimer and tetramer structures could be generated with the**

**TABLE 2**

**Data collection and refinement statistics**

| Entry          | Apo-TnDhp + ADA | Mono-Zn TnDhp | Di-Zn TnDhp | Di-Zn TnDhp + NCβ1 | Di-Zn TnDhp + NCβA | Di-Zn TnDhp + hydantoina |
|----------------|-----------------|--------------|-------------|-------------------|-------------------|-------------------------|
| Data collection |                 |              |             |                   |                   |                         |
| Wavelength (Å)  | 1.000           | 1.000        | 1.000       | 1.000             | 1.000             | 1.000                   |
| Temperature (K) | 110             | 110          | 110         | 110               | 110               | 110                     |
| Space group     | I4122           | I4122        | I4122       | I4122             | I4122             | I4122                   |
| a              | 160.82          | 160.99       | 160.48      | 161.20            | 161.08            | 161.03                  |
| b              | 160.82          | 160.99       | 160.48      | 161.20            | 161.08            | 161.03                  |
| c              | 94.55           | 93.4         | 93.30       | 93.80             | 94.53             | 93.44                   |
| Resolution (Å)  | 30–1.95 (2.1–1.95) | 30–2.0 (20.3–2.00) | 30–1.9 (1.97–1.90) | 30–1.8 (1.86–1.80) | 30–2.0 (2.07–2.00) | 30–2.2 (2.28–2.00) |
| Completeness (%) | 99.6 (100)     | 99.2 (99.6)  | 99.7 (99.9) | 99.8 (100)       | 100 (100)        | 97.6 (99.4)             |
| Redundancy4    | 9.7 (9.5)       | 3.3 (3.2)    | 8.4 (8.3)   | 4.6 (4.5)         | 5.0 (5.0)        | 4.5 (4.4)               |
| Rwork/Rfree (%) | 45.6 (30.2)    | 5.6 (33.5)   | 4.8 (49.9)  | 6.9 (34.1)        | 7.6 (33.9)       | 7.6 (38.0)              |
| No. of atoms   | 3805            | 3759         | 3759        | 3759              | 3759              | 3759                    |
| Protein        | 39 (ADA)a       | 1            | 2           | 10                | 9                 | 7                      |
| Zinc           | 232             | 1            | 2           | 2                 | 2                 | 2                      |
| Water          | 232             | 2           | 216         | 247              | 200              | 170                    |
| B-factors (Å²) | 18.92           | 28.69        | 18.99       | 24.97             | 25.18             | 29.67                   |
| Protein        | 39.1            | 37.93        | 37.93       | 26.34/26.86       | 33.7/39.6        | 39.5/50.1               |
| Zinc           | 23.21           | 32.13        | 22.92       | 30.30             | 30.14             | 33.50                   |
| Water          | 2041            | 1.927        | 1.763       | 2.143             | 1.867             | 1.729                   |

a Values in parentheses are for the highest resolution shell.

b Rfree = 2 [\(\sum h_i(|F_{o}|^2 − |F_{c}|^2)/\sum h_i |F_{o}|^2\)], where \(h_i\) is the ith measurement and \(\langle |F_{o}|^2\rangle\) is the weighted mean of all measurements of \(|F_{o}|^2\).

c Rwork = \(2 \sum h_i (|F_{o}|^2 − |F_{c}|^2)/\sum h_i |F_{o}|^2\), where \(F_{o}\) and \(F_{c}\) are the observed and calculated structure factor amplitudes of reflection \(h_i\).

d Rfree is as Rwork but calculated with 10% of randomly chosen reflections omitted from refinement.

![FIGURE 1. Reactivation of the apo-TnDhp with zinc ions.](image-url)
space group I4₁2₂ (Fig. 2, C and D), which exhibits molecular packing similar to that of other previously reported tetramers (39). The results of the gel filtration during protein purification suggest that the TnDhp is a tetramer in the solution state. Interestingly, the dimeric interface of TnDhp shows that the long C-terminal tail (Val⁴⁷⁸–Ala⁴⁹₅) from one monomer winds onto the surfaces of another monomer and reaches close to its active site. Several hydrogen bonds stabilize the dimeric interaction through the C terminus.

The Metal-binding Site and Post-translational Modification of Lysine by Carbamylation—The metal-free TnDhp (apo-TnDhp) was generated, and the metal content (~0) was confirmed by ICP-MS (Table 1). The active site of apo-TnDhp contains four histidines (His⁶₃, His⁶₅, His¹₈₈, and His²₄₄), one aspartic acid (Asp²₂₂), and one lysine (Lys¹₅₅) in the catalytic pocket (Fig. 3A). In the structure of the apo-TnDhp, the residue of Lys¹₅₅ is not carbamylated at the ε-amino group. Two water molecules, but no metals, occupy the potential metal-binding sites.

The mono-Zn TnDhp contains one metal at the active site, as confirmed by ICP-MS assays (Table 1). The zinc-anomalous difference Fourier map clearly indicates only one zinc atom in the active site (defined as the Mα site) (Fig. 3B). This zinc ion is coordinated with residues His⁶₃, His⁶₅, and Asp²₂₂, with zinc to nitrogen distances of 2.2₁, 2.1₆, and 2.1₃ Å, respectively. Two water molecules are also observed. One water molecule is loosely associated with the zinc atom at a distance of 4.4 Å. The other water is directly coordinated (2.2 Å) to Tyr¹₆⁹, which is the proposed key residue participating in the catalysis (39). The composite omit map shows that the electron density between the Mα metal and Lys¹₅₅ is connected. This electron density is consistent with the carbamate of a carbamylated lysine with a reasonable distance of 2.1₄ Å between one of the oxygens of the carbamate and the Mα metal. An inspection of 10 data sets, which were collected independently, reveals the same result as described above. The structure of mono-Zn TnDhp shows that one metal (Mα) is sufficient to promote the carbamylation process. Evidently, Lys¹₅₅ is chemically modified, with one metal ion at the active site of the protein.

Sequence alignment as well as the structural features of the active site uncovered above suggest that the catalytic pocket of TnDhp could potentially accommodate two metal ions. To generate the di-Zn TnDhp, we soaked crystals of the mono-Zn TnDhp in the crystallization milieu containing zinc ions at high concentrations (0.1 mM). The structure of the di-Zn TnDhp shows two zinc ions at the active site (Fig. 3C), of which one zinc atom is located at the same Mα site as in the mono-Zn structure, and the other, dubbed the Mβ site, is bound to the two histidine residues His¹₈₈ and His²₄₄ with zinc to nitrogen distances of 2.1₅ and 2.1₃ Å, respectively. The distance between the two zinc atoms (i.e. Mα and Mβ) is 3.7₄ Å in the di-zinc TnDhp. The existence and position of the second zinc was confirmed by zinc-anomalous diffraction. These results indicate that the active site could accommodate a total of two zinc atoms. A water molecule is also found at the position between the two zinc atoms (Fig. 3C). In the dimetal TnDhp, each of the zinc ions is coordinated with one of the carba-
mate oxygens of the carbamylated Lys$^{155}$ with a zinc to oxygen distance of 1.97 Å.

Superimposed structures of the apo-TnDhp and the mono- and di-Zn TnDhp reveal conformational changes upon lysine carbamylation and metal binding (Fig. 3D). In the surrounding environment of the active site, the side-chain conformations of His$^{63}$, His$^{65}$, and His$^{244}$ are relatively stable, whereas those of Tyr$^{160}$, His$^{188}$, and Asp$^{322}$ are altered. Relative to their positions in the apo-TnDhp structure, we find that Tyr$^{160}$, His$^{188}$, and Asp$^{322}$ have shifted their side-chain orientations by distances of ~1.9, 0.5, and 0.3 Å, respectively, upon binding of one metal and carbamylation in the mono-Zn structure. After carbamylation and the binding of the two zinc ions, these residues move closer to the metal sites, and the movements increase to 2.0, 0.8, and 0.6 Å, respectively.

The conformation of the Lys$^{155}$ is also greatly affected by the metal binding and carbamylation (Fig. 3D). In apo-TnDhp, Lys$^{155}$ exhibits the general non-carbamylated conformation. Upon zinc binding to the Mo site, the post-modification process takes place by stabilizing CO$_2$ already on the ε-amino group of Lys$^{155}$. It is of interest to note that the free oxygen atom of the carbamate in the mono-Zn TnDhp structure is located at the Mβ position coordinating with His$^{244}$ (2.4 Å). Moreover, the side chain of the carbamylated Lys$^{155}$ has shifted away 1.4 Å to yield space for the second zinc atom to fit into the Mβ site in the structure of the di-Zn TnDhp.

It has been reported that cobalt atoms could substitute for zinc at the active site (40). We have confirmed that the structures of the mono-Co and di-Co TnDhp are similar to the zinc structures described above.

Comparison of Active Sites of Carbamylated Proteins—A protein generally contains several lysine residues, but carbamylation is typically found at special positions, which is extremely interesting and important. The propensity toward carbamylation must depend on the environment of the lysine, including the surrounding residues. The carbamylated lysines are usually buried within the protein structures with a small solvent-accessible surface area, ~6.2 Å$^2$, as in apo-TnDhp, and are surrounded by hydrophobic residues, such as Val and Phe. At present, there are about 250 structures in the Protein Data Bank containing carbamylated lysines required for their functions. These structures could be classified into three groups based on the metal content: 1) no metal; 2) carbamylated lysine with one metal in “half-occupied” geometry; and 3) carbamylated lysine with two metals in full geometry (Scheme 1).

The structures of TnDhp reveal a common structural motif for lysine carbamylation with both Mo and Mβ sites. This motif has been demonstrated to be important for maintaining the carbamate structure according to site-directed mutagenesis and activity rescue experiments (40). One metal is bound to the Mo site sharing two histidines. Apparently, this metal coordination is sufficient to fix CO$_2$ to the lysine. Interestingly, there are several other systems, such as transcarboxylase (Protein Data Bank entry 1RQB) (41), pyruvate carboxylase (2QF7) (6), and pyruvate carboxylase (3BG3) (7), with the same “half-occupied” geometry utilized by the respective lysine to coordinate one cobalt, zinc, and manganese ion, respectively (Fig. 4). A carbamylated lysine with one metal has also been reported in Rubisco (4). The two highly conserved histidine residues near the carbamylated lysine exist also in Rubisco, although the
metal is bound to the ribulose 1,5-bisphosphate substrate instead of the histidines. Thus, our mono-Zn TnDhp provides a new structural feature for enzymes that contain only one tightly bound metal in full geometry, differing from those structures in the Protein Data Bank.

A ligand structure comprising four histidines and one aspartic acid utilizing both the M\textsubscript{H9251} and M\textsubscript{H9252} sites is required for lysine carbamylation and other biological functions mediated by this family of proteins (Fig. 4B). Examples include the amidohydro-lase superfamily (e.g. urease (42), allantoinase (43), HYD (44), and DHP (5)) and other proteins, such as arginine carboxypeptidase (45).

Lysine Carbamylation and Metal Numbers Regulate the Structure and Function of the Protein—The post-translational lysine carbamylation facilitates additional metal coordination to trigger the observed structural alteration seen in our structures for specific enzymatic activities. The molecular structure of apo-TnDhp also reveals a tunnel from the surface of the protein to the active site suitable for the substrate entrance or product release (Fig. 5B). Notably, two loops located at the entrance of the tunnel and near the active site are highly dynamic (Fig. 5A). One loop from Ala\textsubscript{69} to Arg\textsubscript{74} exhibits the largest variation among three structures (apo-, mono-Zn, and di-Zn TnDhp). In particular, Phe\textsubscript{70} and Met\textsubscript{71} swing with a maximum range of 6.9 and 7.5 Å, respectively. The other loop from Met\textsubscript{158} to Met\textsubscript{165} also exhibits a similar structural disparity, allowing Tyr\textsubscript{160} to move closer to the active site in the dimetal structure with a displacement of 2 Å.

In the structure of apo-TnDhp, the two loops are positioned away from the active site with distances of 12.5 and 5.7 Å measured from Phe\textsubscript{70} (on the first loop) and Tyr\textsubscript{160} (on the second loop) to M\textsubscript{H9251} and M\textsubscript{H9252}, respectively. We surmise that this is the “open form” of the enzyme because there is no apparent barrier restricting the transport of the substrate from the surface to the active site (Fig. 5B). The bottleneck dimension of the tunnel in an open form is about 10 Å (Fig. 5D), which is greater than the average size (≈6 Å) of the TnDhp substrates, such as DHU and DHT. However, the two dynamic loops in di-Zn TnDhp move closer to the active site with distances of 8.5 and 4.5 Å from Phe\textsubscript{70} and Tyr\textsubscript{160} to M\textsubscript{158} and M\textsubscript{165}, respectively, and significantly reduce the bottleneck dimension to 3 Å (Fig. 5, C and E), resulting in a “closed form” of TnDhp and limiting the substrate entry into or product release from the active site.

Interestingly, in the structure of the mono-Zn TnDhp, the orientation of the (Ala\textsubscript{69}–Arg\textsubscript{74}) loop is in the “intermediate state” between the conformations of apo- and di-Zn TnDhp.
The dynamic loops in the mono-Zn structure are about 40% in the open form and 60% in the closed form based on the electron density analysis. The averaged temperature B-factor of this loop (Ala$^{69}$–Arg$^{74}$) in the mono-Zn TnDhp ($\sim$60.2 $Å^2$) is higher than that of either the apo-TnDhp ($\sim$32.2 $Å^2$) or the di-Zn TnDhp ($\sim$26.0 $Å^2$), suggesting that the (Ala$^{69}$–Arg$^{74}$) loop has the propensity to undergo structural fluctuations even in the crystalline state.

It has been reported that the d-HYD from *Bacillus stearothermophilus* is equipped with three stereochemistry gate loops (SGL) located in the tunnel gate to regulate the substrate specificity and activity (46, 47). The first (Ala$^{69}$–Arg$^{74}$) and second (Met$^{158}$–Met$^{165}$) dynamic loops found in TnDhp are structurally related to the gate loops SGL1 and SGL3, respectively, in *B. stearothermophilus* d-HYD. In this study, we have provided evidence for structural or conformational fluctuations of similar SGLs in vertebrate dihydropyrimidase through a comparison of the apo- and metal-binding structures.

**The Flexible C-terminal Tail**—The role of the C terminus in DHP has been investigated for a long time, but its function is still a mystery. It has been suggested that the C terminus may play a role in maintaining a multimer form of proteins. It is known that the deletion of the C-terminal fragment in D-HYD results in dissociation of the protein from dimers to monomers (48). In our structure of TnDhp, the C-terminal tail also maintains the dimeric form, with its terminus reaching near the dynamic (Ala$^{69}$–Arg$^{74}$) loop of the active site (Figs. 2C and 5B). An inspection and comparison of the C-terminal tails in apo-, mono-Zn, and di-Zn TnDhp structures show that the stability of the C-terminal tail, especially the fragment from Pro$^{488}$ to Ala$^{495}$, affects the dynamic (Ala$^{69}$–Arg$^{74}$) loop (Fig. 5A). The structure of the C-terminal fragment (Pro$^{488}$–Ala$^{495}$) is rigid, with well-defined electron densities in the apo-TnDhp structure (the open form) (Fig. 5B), whereas it is flexible in the monometal structure (an intermediate state). In the latter, the electron density of this region is diminished so that model building could only be allowed with the density map at a lower contour level (0.7 $\sigma$). Finally, in the di-Zn TnDhp structure (the closed form), the electron density of the C-terminal tail is too indistinct to allow model building in this region of the protein structure (Fig. 5C). Thus, the flexibility of the C-terminal tail and the conformation of the dynamic loops appear to be correlated. Note that a small C terminus fragment of five residues after Ala$^{495}$ is too flexible to be identified in all of the structures, so we could not exclude the possibility that these five residues are also interacting with the first dynamic loop.

**Structures of TnDhp in Complexes with Hydantoin, NCβI, and NCβA**—To explore the substrate-binding site and probe the interactions between the substrates and the residues essential for catalysis, we have co-crystallized TnDhp in complexes with hydantoin, DHT, and DHU, separately. Although both mono-Zn and di-Zn TnDhp are prepared for co-crystallization, only the structures of the complexes with two zinks in the active site could be successfully obtained (Fig. 6). This interesting result might reflect the fact that the substrate channel of the intermediate state in mono-Zn TnDhp does not contain barriers to restrict the diffusion of substrate and products to and...
from the active site. The hydantoin is found in the catalytic pocket (Fig. 6A) and interacts with two zinc atoms (with distances of 2.8 and 3.0 Å), the main chain of residue Gly294 (3.8 Å), and the side chains of Tyr160 (3.8 Å) and Asp322 (3.4 Å). Phe70, which is located in the dynamic loop (Ala69–Arg74), described above, is in van der Waals contact with the hydantoin (3.8 Å) (Fig. 5A) and stabilizes the substrate via - stacking interactions.

From the co-crystallization experiments with the DHT and DHU, it is clear from the electron densities that the C4–N3 bonds in these substrates have been broken to open up the dihydropyrimidine ring and generate the corresponding products NCβI and NCβA, respectively. These observations indicate that catalytic cleavage of the substrates has taken place, but the products could not be released with the two zinc atoms present in the active site (Fig. 6, B and C). Several residues are utilized for stabilizing the NCβI as well as the NCβA, as listed in Table 3. The distances from Tyr160 to the N3 and O4 atoms of NCβI are 3.0 and 2.7 Å, and the distances to the N3 and O4 atoms of NCβA are 3.1 and 2.3 Å, respectively. From the structure of the NCβI in complex with the TnDhp, we conclude that the stereo-selectivity of the catalysis is the L-conformation of the substrate.

Activity assays indicate that the TnDhp processes hydantoin with a low activity (~330-fold lower compared with DHT or DHU), in agreement with our structural results indicating that the hydantoin is maintained in the cyclic form, but both DHT and DHU have been hydrolyzed in the structures of the complexes. The positions of the NCβI and NCβA are similar in the structures (Fig. 6D), with the cutting sites pointing toward Tyr160. However, the cutting site of hydantoin is displaced ~2.5 Å away from Tyr160 compared with DHT or DHU, which may explain the low activity of the di-Zn TnDhp toward hydantoin.

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the active site (Fig. 7), highlighting the potential pathway(s) for substrate entry and product release. One ADA molecule (ADA3) is coordinated with the side chains of His\(^{231}\) and Asn\(^{343}\) and the main chains of Phe\(^{338}\) and Thr\(^{339}\). A second ADA (ADA2) is located in the middle position and interacts with the side chains of Tyr\(^{160}\) and Asn\(^{343}\) and the main chains of Thr\(^{339}\), Ile\(^{341}\), and Asn\(^{343}\). The residue Tyr\(^{160}\) in Tn\(Dhp\) is structurally equivalent to Tyr\(^{152}\) in Sinorhizobium meliloti DHP, which has been proposed to be associated with the catalysis process by a mutagenesis study (39). The third ADA (ADA1) at the position close to the active site is coordinated with the side chain of Asp\(^{322}\) and main chain of Gly\(^{294}\). Notably, this ADA1 molecule is located at the same position with an orientation comparable with that in the substrates hydantoin, NC\(^{1092}\)I, and NC\(^{1092}\)A described earlier in the structures of the substrate complexes (Fig. 7B). In addition, several hydrogen bonds mediated by water molecules are found to stabilize ADA molecules between residues and ADA molecules. The detailed interactions with the corresponding distances are listed in Table 3.

DISCUSSION

**Structural Insights into the Stages of Lysine Carbamylation**—The structures with varying metal contents provide insights into the different stages of lysine carbamylation (Scheme 1). The first stage is depicted by apo-\(TnDhp\), where Lys\(^{155}\) is not carbamylated. The second stage (precarbamylation) might involve the formation of an intermediate with a carbon dioxide weakly associated with the \(-NH_2\) group of the lysine (49). This structure is expected to be quite labile, so that the process is reversible. The third stage corresponds to the carbamylation of the lysine, culminating in the structure of the mono-Zn \(TnDhp\), in which the lysine has been carbamylated at the active site with only one metal. Although carbon dioxide exists at a concentration of \(~50\) ppm in the solution, and reversible carbamylation on Lys\(^{155}\) (Stage II of Scheme 1) can be expected, one zinc or cobalt ion must be positioned at the active site for CO\(_2\) fixation (Stage III of Scheme 1). The final stage is represented by the structure of the di-Zn \(TnDhp\), where a second metal ion has bound to the active site to stabilize the carbamylated lysine.

The Potential Role of the M\(_2\) Metal Ion—Beside the catalytic metal ion at the M\(_\alpha\) site, the active site of \(TnDhp\) allows binding of a second metal ion for regulatory function. Enzymes that equip the active site with full geometry as well as a second metal ion for regulatory function are observed in the amidohydrolase superfamily (50). It has also been reported that zinc ions at high concentrations could enhance the thermal stability of DHP (51). A structural comparison of apo-\(TnDhp\) and mono- and dimetal \(TnDhp\) suggests that the metal ion at the M\(_\beta\) site regulates the size of the substrate tunnel (Fig. 5). Presumably, the M\(_\beta\) metal site plays the role of controlling the two dynamic loops (Ala\(^{69}\)–Arg\(^{74}\) and Met\(^{158}\)–Met\(^{165}\)) for substrate entrance, substrate locking, and product release. Upon the substrate entering the active-site pocket, the M\(_\beta\) metal ion is simultaneously positioned at the M\(_\beta\) site to alter the conformations of the dynamic loops to the “closed form” and potentially lock the substrate at the right position with Phe\(^{70}\) for catalysis. After initiating the catalytic turnover, the M\(_\beta\) metal ion would of
course ultimately have to leave the Mβ site to generate an “open-form” substrate tunnel for the product release and new substrate entrance. Thus, the weak metal binding exhibited by the Mβ site is obligatory for its regulatory function, making the occupancy of the Mβ site by a metal ion observable only upon soaking crystals of the mono-Zn TnDhp with zinc ions at a high concentration. This phenomenon might resolve the puzzle as to why only one metal was detected previously in vertebrate DHPSs with the full geometry (26, 52). In the normal enzymatic state, the Mα metal ion is tightly bound for catalysis, but the binding of metal to the Mβ site is weak in order to provide the protein with dynamic features to control the “open/closed” conformations of the two dynamic loops. It has been proposed that the metal ion at the Mβ site is loosely bound in DHP (5).

The Importance of Protein Oligomerization—The dimerization of TnDhp results in the C-terminal tail from one monomer reaching near the dynamic (Ala⁶⁹–Arg⁷⁴) loop of another monomer (Figs. 2C and 5). This feature of the structure allows us to probe the function of the C terminus. The structural analysis reveals that the conformational stability of the C-terminal tail is in the following order: apo-TnDhp > mono-Zn TnDhp > di-Zn TnDhp. It seems that the flexibility of the C-terminal tail is correlated with the conformations of the dynamic (Ala⁶⁹–Arg⁷⁴) loop. The open form of the dynamic loop is linked to the greater stability (low flexibility) of the C-terminal tail, whereas the low stability (high flexibility) of the C-terminal tail results in a closed form of the dynamic loop, implying that the protein is exploiting the flexibility of the C-terminal tail to control the size of the substrate tunnel. Therefore, our structural analysis indicates that the C-terminal tail dictates not only the dimerization of the protein but also the enzymatic activity. It is possible that dimerization of TnDhp by the C-terminal tail assists the opening of the two dynamic loops (SGL1 and SGL3) for the enzyme function.

Previous reports have shown that modifications to the C termini of DHP/HYD enzymes could decrease the enzyme activity (9, 48, 53–55). In the d-HYD from B. stearothermophilus and Bacillus thermocatenulatus, the truncated enzymes with a deletion of 40 amino acids on the C terminus have lost 64% of their enzymatic activities (48). In Pseudomonas putida YZ-26, a series of deletions were performed in the d-HYD and all of the truncated enzymes exhibited either low or non-detectable activities (53, 54). However, CD spectroscopy showed that these deletions of the C-terminal fragment did not alter the secondary structures of the overall folding (48, 54).

More recently, deletions on the C terminus of DHP from human, a vertebrate similar to T. nigroviridis, have been undertaken, and these mutants also show no detectable activity (9). Evidently, the C-terminal tail plays an important role for the activity of the enzyme.

Proposed Structure-based Mechanism; Monometal Active Site—According to our structures of apo-, monometal, and dimetal TnDhp, substrate complexes, and the ADA complex, we have gained important insights into the mechanism of action of the DHP family of enzymes. The first step of the catalytic turnover is to get the substrate into the active site. This state is highlighted by the apo-TnDhp and monometal TnDhp structures with the open form of the tunnel, which provides a suitable cross-section for the substrate to diffuse into the active site (Figs. 5D and 8A).

In order to maintain the substrate in the specific position for catalysis, some structural changes have occurred. Two dynamic loops (Ala⁶⁹–Arg⁷⁴ and Met¹⁵⁸–Met¹⁶⁵) with aromatic residues Phe⁷⁰ and Tyr¹⁶⁰ have moved closer to the active site, locking the substrates in the active site, as seen in the dimetal TnDhp with the tunnel characteristic of the closed form (Figs. 5E and 8B).

While the substrate is bound to the active site, some residues, such as Gly²⁹⁴ and Asn⁴⁴³, are employed to stabilize the substrate. For substrates, such as DHT, that belong to 5-monosubstituted dihydropyrimidines, the stereo-selectivity of DHP is the L-configuration, not the D-configuration, as seen in the structure of the NCβl complex (Fig. 6B).

Catalytic mechanisms involving both one zinc and two zinc ions have been proposed for the DHP proteins (5, 37, 55). The mechanism with one zinc ion was first proposed for the DHP from calf and pig liver (55). In this proposed mechanism, the zinc ion acts as a Lewis acid, and an amino acid residue behaves both as a general base and acid to mediate the catalysis. The substrate is bound to the metal ion (Lewis acid) through the 4-oxo group; the general base, most likely the aspartic acid (Asp³²₂ in TnDhp), activates a water molecule for the nucleophilic attack at the C-4 carbon of the substrate, generating a tetrahedral intermediate, which is stabilized by a tyrosine residue (Tyr¹⁶⁰ in TnDhp) (5). The ring of the substrate in the tetrahedral intermediate then opens up to yield the products upon general acid protonation of the ring nitrogen using the same aspartic acid residue. On the other hand, the two-zinc mechanism of DHP proposed is based on the mechanism of dihydroorotase (24, 38), because it is generally believed that these two protein systems share the similar catalytic mechanism (5, 39). In this scheme, the Mß zinc interacts with the carbonyl oxygen (O4) of the substrate. This is followed by nucleophilic attack through the hydroxyl group of the water molecule and assisted by the carboxylate of the aspartic acid. Both the Mß and Mß zinc ions as well as a tyrosine residue (Tyr¹⁶⁰ in TnDhp) (5) are used to stabilize the tetrahedral intermediate state. After protonation of the amide nitrogen of the substrate assisted by the same aspartic acid, the product is generated and coordinates with the two zinc ions.

Indeed, our activity and structure analyses of TnDhp described earlier show that one stable metal in the active site could achieve the maximum activity. Thus, it seems that the one-metal mechanism is operative in the catalysis mediated by the vertebrate DHP. However, according to our results, the metal in the Mß site is only loosely bound in the absence of substrate. The regulatory role we discussed earlier for this metal could well be related to the binding of substrate and subsequent catalysis (Fig. 8C). Hence, it might be that only one metal is detected in the active site in vertebrate DHPSs, but for the biochemistry, the second metal is essential, and it is conceivable that the binding of the second metal and of the substrate reinforce each other to promote the catalysis. On the other hand, as a ligand of the Mß metal, the role of Asp³²₂ as a general base/acid is diminished. Instead, Tyr¹⁶⁰ stabilizing the intermediate state may be important in the catalysis turnover.
After the catalytic reaction, the product can only be released through the same tunnel. To change the tunnel from the closed to the open form, extra forces will be needed to entice the dynamic loops to return to the open state. According to our structures, the C-terminal tail from the other monomer is positioned near the dynamic loop and the active site. This C-terminal region has high temperature B-factors in the x-ray structure, so the dynamic loop could potentially open via thermal fluctuations (Fig. 5, B and C). While the dynamic loop is open, the product (and possibly the second metal as well) could diffuse out of the active site, following the pathway(s) of the three ADA molecules in the tunnel of the apo-TnDhp structure (Figs. 7 and 8D). Subsequently, another cycle of the reaction can be repeated with a new substrate entering into the active site.

In summary, we have demonstrated the possible existence of four stages of the metal-mediated lysine carbamylation with various conformational changes at the active site for the biological function of a vertebrate DHP. Lysine carbamylation results in a tightly bound metal ion at the Mα site, which participates in both CO2 fixation and catalysis, and a weakly bound metal ion at the Mβ site introduces specific dynamic properties into the protein structure to allow the regulation of the size of the substrate/product tunnel. This information provides a basis to explain the longstanding question in the literature on the varying number of metal ions associated with the carbamylated lysine in this class of enzymes.

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