Purification, Characterization, and Inhibition of Peptide Deformylase from *Escherichia coli*†

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ABSTRACT: Peptide deformylase (EC 3.5.1.31) catalyzes the removal of a formyl group from the N-termini of nascent ribosome-synthesized polypeptides, an obligatory step during protein maturation in eubacteria. Since its discovery in crude *Escherichia coli* extracts 3 decades ago, the deformylase has resisted all attempts of purification or characterization due to its extraordinary lability. By placing the coding sequence (*def* gene) of *Escherichia coli* deformylase behind a bacteriophage T7 promoter, we have, however, been able to overexpress this deformylase in *Escherichia coli*. Overproduction has allowed the purification of >50 mg of deformylase enzyme from each liter of cell culture. Purified deformylase is highly active toward N-formylated peptide substrates. A new, sensitive assay for the deformylase has been developed by measuring the amount of released formate using a formate dehydrogenase. This has allowed for the assessment of the catalytic properties of peptide deformylase using a series of synthetic N-formylated peptides as substrates. The deformylase exhibits strong preference for an L-methionine or the isosteric norleucine at the N-terminus of a substrate and has broad specificity for the rest of the residues. Small divalent metal chelators strongly inhibit the *E. coli* deformylase. In particular, certain 1,2- and 1,3-dithiol compounds act as potent, time-dependent inhibitors of the peptide deformylase.

Ribosome-mediated synthesis of proteins starts with a methionine residue. In prokaryotes and eukaryotic organelles (mitochondria and chloroplasts), the methionyl moiety carried by the initiator tRNA is N-formylated prior to its incorporation into a polypeptide [reviewed by Meinnel et al. (1993)]. Consequently, *N*-formylmethionine is always incorporated at the N-terminus of a nascent polypeptide (Adams & Capecchi, 1966; Webster et al., 1966). However, most mature proteins do not retain the *N*-formyl group (Marcker & Sanger, 1964). Moreover, in a cytosolic extract of *Escherichia coli*, only 40% of the polypeptides retain the N-terminal methionine (Waller, 1963). Instead, the majority of the polypeptides display alanine, serine, or threonine at their N-termini, indicating that the *N*-formylmethionine group is removed post- or co-translationally.

Deformylation is a part of the methionine cycle involved in protein biosynthesis (Figure 1), which consists of five enzymes: methionyl-tRNA synthetase (MTS), methionyl-$tRNA^{Met}_f$ formyltransferase (MTF), peptide deformylase (PDF), methionine aminopeptidase (MAP), and peptidyl-tRNA hydrolyase (PTH) [reviewed by Meinnel et al. (1993)]. Methionyl-tRNA synthetase catalyzes the addition of a methionine to the 3′- or 2′-OH group of the terminal adenosine in $tRNA^{Met}$ (Heinrikson & Hartley, 1967). The formyltransferase, MTF, then transfers a formyl group from N^{10}-formyltetrahydrofolate to the NH_2 group of methionine on Met-$tRNA^{Met}_f$ (Adams & Capecchi, 1966; Dickerman et al., 1967; Kahn et al., 1980; Blanquet et al., 1984; Guillon et al., 1992). Following the translational initiation, the peptide deformylase cleaves the formyl group from the nascent polypeptide chain (Adams, 1968; Livingston & Leder, 1969; Takeda & Webster, 1968). The methionyl aminopeptidase then removes the N-terminal methionine from certain deformylated peptides to produce mature proteins (Ben-Bassat et al., 1987; Miller et al., 1987), depending on the identity of the penultimate residue (Hirel et al., 1989). Occasionally, protein synthesis aborts prematurely; in such an event, the peptidyl-tRNA hydrolase cleaves the peptide moiety from immature peptidyl-tRNAs in order to recycle the tRNAs (De Groot et al., 1969).

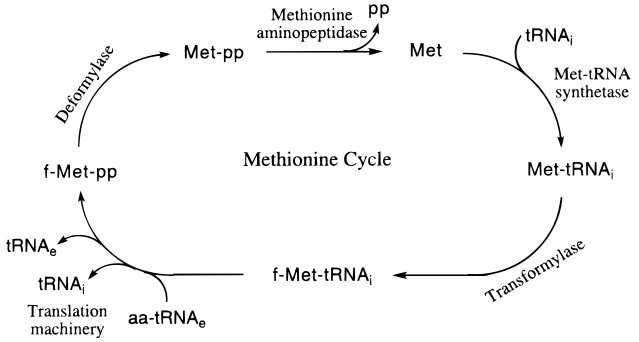

FIGURE 1: Methionine cycle of eubacteria. pp, polypeptide; aa, amino acid; f, formyl; $tRNA_i$, initiator tRNA; $tRNA_e$, tRNAs for peptide elongation.

Among the enzymes involved in the methionine cycle, peptide deformylase is least well characterized, because its extraordinary lability makes its isolation exceedingly difficult. It was reported that the deformylase was rapidly inactivated upon exposure to low-molecular-weight sulfhydryl molecules or dialysis conditions (Adams, 1968; Livingston & Leder, 1969). Even mild procedures such as Sephadex G-100 or DEAE-cellulose chromatography or ultracentrifugation on sucrose gradient resulted in almost quantitative loss of enzymatic activity (Adams, 1968). The lack of pure, active protein has so far prevented any systematic study of the

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mechanism or kinetic properties of this enzyme. Recently, the deformylase gene (*def* or *fms*) was cloned from *Escherichia coli* (Mazel et al., 1994) and *Thermus thermophilus* (Meinnel & Blanquet, 1994). The deformylase has also been overexpressed in *E. coli* and purified to apparent homogeneity (Meinnel & Blanquet, 1993). However, the purified deformylase protein had no catalytic activity. Although the protein was later shown to possess some deformylase activity, the activity was very low ($k_{cat}/K_M \sim 80\ M^{-1}\ s^{-1}$ with formyl-Met-Ala-Ser as substrate) (Meinnel & Blanquet, 1995).

In this paper, we describe an efficient overexpression and purification procedure which produces >50 mg of pure, highly active *E. coli* peptide deformylase ($k_{cat}/K_M \sim 10^4-10^6\ M^{-1}\ s^{-1}$) from a liter of cell culture. A new, sensitive assay for peptide deformylase has been developed. The catalytic properties of deformylase toward a series of formylated peptides have been determined. We also show that certain divalent metal chelators and dithiols are potent inhibitors of peptide deformylase. In the preceding paper (Chan et al., 1997), we have determined the three-dimensional structure of the *E. coli* enzyme by X-ray crystallography, which shows significant differences from the structure reported for the less active deformylase (Meinnel et al., 1996).

EXPERIMENTAL PROCEDURES

Materials. All Fmoc-protected amino acids, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and 1-hydroxybenzotriazole (HOBT) were purchased from SynPep (Dublin, CA). HMP resin was from Applied Biosystems. Formate dehydrogenase and *Aeromonas* aminopeptidase were from Sigma. Other chemicals including ethanedithiol and its derivatives, β-mercaptoethanol, 1,10-phenanthroline, iodoacetate, and 4-(chloromercuri)benzoic acid were purchased from Aldrich and used directly in assays.

Buffers. Buffer A, 50 mM Hepes, pH 7.0, 10 mM NaCl, and 1% Triton X-100; buffer B, 20 mM sodium phosphate, pH 7.0, 10 mM NaCl; buffer C, 10 mM Bis-Tris, pH 7.0, 10 mM NaCl; buffer D, 50 mM sodium phosphate, pH 7.0, 10 mM NaCl, and 1 mM EGTA; buffer E, 50 mM Hepes, pH 7.0, 10 mM NaCl; buffer F, 50 mM Hepes, pH 7.0, 100 μg/mL bovine serum albumin.

Expression and Purification of Deformylase. *E. coli def* gene was isolated by a polymerase chain reaction (PCR) using two primers, 5′ GGAAGGCCATATGTCAGTTTTGCAAGTG 3′ and 5′ GGGAATTCTGACACGTTAGTTCT 3′, which are complementary to the sequences at the 5′ and 3′ termini of the *def* gene, respectively (Mazel et al., 1994). A single colony of *E. coli* BL21(DE3) cells was transferred into 50 μL of TE buffer (10 mM Tris·HCl, pH 8.0, 1 mM EDTA), boiled at 100 °C for 5 min, and used directly in PCR as the DNA template. PCR (100 μL total volume) was carried out in a solution containing 1× Vent DNA polymerase buffer (New England Biolabs, Beverly, MA), the DNA template, dNTPs (250 μM), 5′ and 3′ primers (1 μM each), and 2.5 units of Vent DNA polymerase (New England Biolabs). The reaction was allowed to proceed for 30 cycles: 1 min at 94 °C, 1 min at 52 °C, and 1 min at 72 °C. The PCR product was digested with the restriction enzymes *Nde*I and *Eco*RI, which recognize sequences built into the PCR primers. The resulting DNA fragment, ~500 base pairs in length, was inserted into the *Nde*I/*Eco*RI cleaved plasmid pET-22b (Novagen, Madison, WI), to produce the desired pET-22b-def construct. The identity of the *def* gene was confirmed by sequencing the entire gene.

E. coli BL21(DE3) cells carrying plasmid pET-22b-def were grown in LB medium supplemented with 75 mg/L ampicillin and 100 μM $ZnCl_2$ at 37 °C. When the OD_{600} reached 0.6, the cells were induced by the addition of 200 μM isopropyl β-D-thiogalactopyranoside (IPTG) at 30 °C for 4 h. Cells (4 L) were harvested by centrifugation and resuspended in 100 mL of lysis buffer A containing 100 μg/mL chicken egg white lysozyme and a protease inhibitor mixture (100 μg/mL phenylmethanesulfonyl fluoride, 20 μg/mL trypsin inhibitor, 20 μg/mL leupeptin, and 20 μg/mL pepstatin). The cells were lysed by incubation for 30 min at 4 °C, followed by sonication for 5 × 10 s pulses. Cell debris was removed by centrifugation at 15 000 rpm for 10 min in a Sorvall SS-34 rotor. The clear supernatant (112 mL) was subjected to ammonium sulfate fractionation, and the 55−80% fraction was dissolved in 10 mL of buffer B and loaded onto a Sephacryl S-300 column (2.5 × 80 cm) equilibrated in buffer B. The column was eluted with ~500 mL of buffer B at 0.7 mL/min. An aliquot of each fraction was analyzed by both SDS−PAGE and deformylase activity assay. Fractions containing the most deformylase activities were pooled (60 mL) and applied onto a Q-Sepharose Fast Flow (Pharmacia) column (2.5 × 7.5 cm) equilibrated in buffer B. The adsorbed molecules were eluted with 150 mL of buffer B plus a linear gradient of 10−380 mM NaCl at 2.0 mL/min. Active fractions (eluted at ~230 mM NaCl) were pooled (30 mL) and precipitated by adjusting to 80% saturation with ammonium sulfate. The precipitate was redissolved in 12 mL of buffer B plus 1.2 M ammonium sulfate and loaded onto a Pharmacia HiLoad 16/10 phenyl-Sepharose column which had been equilibrated in buffer B plus 1.7 M ammonium sulfate. Elution was performed with buffer B and a reverse ammonium sulfate gradient (1.7−0 M; 200 mL at 2.0 mL/min). The active fractions were pooled and concentrated in a Centriprep-10 apparatus (Amicon). Glycerol was added to a final concentration of 33% (v/v), and the enzyme was quickly frozen in liquid nitrogen and stored at −70 °C. Protein concentrations were determined according to Bradford (1976) using bovine serum albumin (Bio-Rad) as standard and corrected according to the equation: actual concn = 0.56 × concn from Bradford (*vide infra*).

Peptide Synthesis. All peptides were prepared by solid-phase synthesis using HMP resin (Applied Biosystems, Inc.) and standard Fmoc/HBTU/HOBT chemistry (Bodanszky, 1993) on a 0.2 mmol scale on a homemade peptide synthesis apparatus. Reactions were monitored by ninhydrin analysis, and the coupling procedure was repeated once when necessary. During the last coupling step, a formyl group was added onto the N-terminus using 96% formic acid, dicyclohexylcarbodiimide (DCC), and dimethylaminopyridine. Cleavage and deprotection were carried out with a mixture of trifluoroacetic acid (4.75 mL), anisole (0.1 mL), phenol (0.1 mg), and ethanedithiol (0.1 mL) for 1 h at room temperature. The solvents were removed by a gentle flow of N_2. The peptides were then triturated 3 times in diethyl ether. After drying in air, the peptides were dissolved in the smallest possible volume of water/acetonitrile/trifluoroacetic acid (50:50:0.1) (~2 mL) and loaded onto a Sephadex G-10 column

(2.5 × 40 cm). The column was eluted with water (plus 0.1% trifluoroacetic acid), and fractions containing the desired peptides were pooled and lyophilized to produce an amorphous powder. HPLC analysis showed generally >90% purity. The identity of the peptides was confirmed by fast atom bombardment mass spectrometry.

Purification of Formate Dehydrogenase. Commercial formate dehydrogenase isolated from *Pseudomonas oxalaticus* (Sigma) contains substantial NADH oxidase activity, which complicates the coupled deformylase assays. The following procedure was employed to remove this contaminating activity. All buffers were degassed exhaustively under vacuum and then sparged with argon or N_2. Crude dehydrogenase powder (~70 mg) was dissolved in 30 mL of buffer C plus 10% (w/v) glycerol and loaded immediately onto a Q-Sepharose column (2.5 × 7.5 cm) which had been equilibrated in buffer C plus glycerol. The bound proteins were eluted with the same buffer plus a linear gradient of 10−800 mM NaCl (2 mL/min). The active dehydrogenase fractions were pooled, concentrated in a Centriprep-10 concentrator, adjusted to 33% glycerol, quickly frozen in 0.5 mL aliquots in liquid nitrogen, and stored at −70 °C. Formate dehydrogenase purified in this fashion has negligible oxidase activity.

Deformylase Assay. Deformylation reactions (total volume of 500 μL) were typically carried out in buffer D or E containing 0−5 mM formylated peptides at room temperature, unless otherwise stated. Reactions were initiated by the addition of 0.1−50 μg of freshly thawed deformylase and allowed to proceed for 30 s. The reactions were then terminated by the addition of 10 mM (final) ethanedithiol and heating to 95 °C for 5 min in a water bath. This procedure completely inactivates *E. coli* deformylase in <5 s. After termination of the deformylase reaction, 10 μL of 100 mM NAD^+ and 440 μL of ddH_2O were added to the reaction. The mixture was transferred into a quartz cuvette, and the dehydrogenase reaction was initiated by the addition of 50 μL of formate dehydrogenase (~100 μg of protein as judged by Bradford assay) purified as described above. The reaction was followed in a Perkin-Elmer λ3 UV−vis spectrophotometer at 365 nm until the formate is depleted (this usually takes 10−30 min). The amount of formate released by the deformylase reaction is determined by comparing the absorbance increase at 365 nm with a standard line generated with sodium formate. For most deformylase reactions, the substrate to product conversion was kept <10%. Enzyme dilutions were made in buffer F. Substrate concentrations (for formylated peptides) were determined by hydrolyzing the formyl group to completion with deformylase or methanolic HCl (Sheehan & Yang, 1958) and measuring the amount of formate released as described above.

pH Profile Analysis. Deformylation reactions were carried out in buffers of various pH values to assess the effect of pH on the catalytic activity of deformylase. The buffers used were 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) for pH 5.0−6.5, 50 mM potassium phosphate for pH 6.5−7.9, and 50 mM 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxy-1-propanesulfonic acid (AMPSO) for pH 8.3 and 8.9. All buffers also contained 10 mM NaCl. The reaction typically contained the appropriate buffer, 800 μM substrate, and 0.8 μg of deformylase.

Inhibition by Effector Molecules. Deformylation reactions (total volume 500 μL) were performed in buffer E containing 800 μM formyl-MA, 1.0 μg of deformylase, and various concentrations (0−1 mM) of effector molecules at room temperature for 30 s. The reactions were terminated by the addition of 200 μM (final) ethanedithiol, and the amount of formate was measured as described.

Inactivation of Peptide Deformylase by Dithiol Molecules as a Function of Time. At time zero, 2.5 μg of deformylase in buffer D was mixed with the dithiol compounds at the specified concentrations in a final volume of 1 mL (at 4 °C). At various times, 100 μL aliquots of this reaction mixture were removed and rapidly diluted into 900 μL of a solution containing buffer D, 100 μM formyl-Met-Leu-*p*-nitroanilide, and 0.4 unit of *Aeromonas* aminopeptidase. The residual activity was determined by following the reaction on a spectrophotometer at 405 nm (Wei & Pei, 1997).

Analytical Gel Filtration. The purified deformylase was injected into a Superdex 75 column connected to a Pharmacia FPLC system. Isocratic elution was carried out in 20 mM sodium phosphate, pH 7.0, 150 mM NaCl at 0.5 mL/min, using RNase A (13.7 kDa), chymotrypsinogen (25.0 kDa), ovalbumin (43.0 kDa), bovine serum albumin (67.0 kDa), and blue dextran (2000 kDa) as standards. The deformylase eluted as a single sharp peak at 24.6 min, corresponding to a molecular mass of 24.5 kDa.

Metal Analysis. Peptide deformylase purified as described above was passed through a Superdex 75 column equilibrated in 10 mM Hepes, pH 7.0, 30 mM NaCl [prepared with metal-free water (Riordan & Vallee, 1988)]. The resulting protein (6.0 mg/mL by Bradford) was analyzed for metal contents using plasma emission spectroscopy at the University of Georgia. Protein-free fractions from the same column were used as the blank. The same protein was subjected to amino acid analysis at Yale University to determine its concentration (3.4 mg/mL). Thus, protein concentration as determined by Bradford assay is corrected by a factor of 0.56. To determine the metal content of ethanedithiol-inactivated deformylase, the enzyme (0.25 mg/mL) was treated with 100 μM ethanedithiol for 60 min at 4 °C and passed through a Superdex 75 column to remove the excess ethanedithiol prior to plasma emission analysis.

RESULTS

Overexpression and Purification. *E. coli def* gene encoding peptide deformylase was cloned by Mazel et al. (1994). Using two synthetic oligonucleotides as primers and *E. coli* genomic DNA as template, the complete coding sequence of the peptide deformylase was obtained by a polymerase chain reaction (PCR). The PCR fragment, after restriction digestion, was inserted into prokaryotic expression vector pET-22b to generate pET-22b-def. *E. coli* cells carrying the plasmid pET-22b-def produce high levels of a 19−23-kDa polypeptide on SDS−PAGE gels (mobility varies depending on the gel percentage). The crude lysate of these cells shows greatly increased deformylase activity compared to uninduced cells or cells which do not carry this plasmid.

The deformylase was subsequently purified to homogeneity by a combination of ammonium sulfate fractionation, gel filtration, anion exchange, and hydrophobic interaction chromatography as described under Experimental Procedures. Typical yields were ~50 mg of deformylase from a liter of *E. coli* culture. The resulting protein shows a single band on SDS−PAGE gels (Figure 2) and is highly active toward

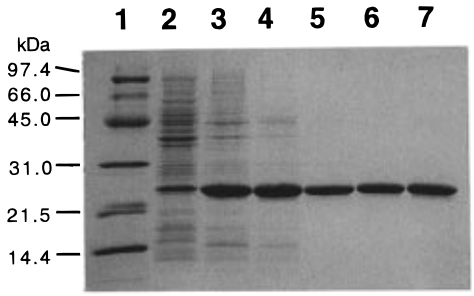


FIGURE 2: Coomassie blue-stained SDS−PAGE gel (15%) showing the purification of peptide deformylase from *E. coli* cells carrying the plasmid pET-22b-def. Lane 1, molecular mass markers; lane 2, uninduced cells; lane 3, crude lysate of induced cells; lane 4, after ammonium sulfate fractionation (55−80%); lane 5, after gel filtration on Sephacryl S-300; lane 6, after Q-Sepharose column; and lane 7, after phenyl-Sepharose column.

N-formylmethionyl peptides. Electrospray ionization mass spectrometry gives a molecular mass of 19 202 ± 20 Da, in good agreement with the 19 197 Da molecular mass predicted from its amino acid sequence (minus the amino-terminal methionine). The specific activity of purified deformylase varied greatly depending on the method of purification. Even different preparations of the enzyme using the same procedure described above gave specific activities that varied by an order of magnitude. All of the kinetic studies in this work were carried out with the most active enzyme batch so far purified.

Physical Properties. The deformylase was reported to be extremely labile in crude cell lysates, with a half-life of 60 s at 37 °C (Adams, 1968; Livingston & Leder, 1969). The purified enzyme shows somewhat improved stability. When stored at 4 °C in a concentrated form (>3 mg/mL), the enzyme remains active after days to weeks; when stored at −70 °C in the presence of 33% glycerol, there is no loss of activity after 2 years. However, the purified deformylase is still a very unstable enzyme. It loses all of its deformylase activity after storage at 4 °C for extended periods of time (>2 months) and can be rapidly inactivated by heating at 95 °C ($t_{1/2} \sim 12$ s). During deformylase assays at room temperature (where the deformylase is usually present at low concentrations), it is inactivated with a $t_{1/2} \sim 2$ min. Therefore, deformylase reactions in this work were generally carried out either for 30 s at room temperature or for <5 min at 4 °C.

It has previously been reported that peptide deformylase is a metalloenzyme, containing one zinc ion per polypeptide (Meinnel & Blanquet, 1993, 1995; Meinnel et al., 1995, 1996). The X-ray crystal structure indicates that a metal ion is present at the putative active site, ligated by three protein ligands and a water molecule (Chan et al., 1997). Plasma emission spectroscopic analyses of our purified deformylase indeed revealed the presence of Zn^{2+} ion, varying from 0.1 to 1.2 Zn^{2+} per polypeptide chain in different enzyme preparations. However, in some enzyme preparations including the one used for kinetic studies in this work, irreproducible but significant amounts (up to 0.3 metal per polypeptide) of iron, copper, and cobalt ions were also observed. Analytical gel filtration on Superdex 75 gave an apparent molecular mass of 24.5 kDa, close to that deduced from its amino acid sequence (19.2 kDa). This suggests that the deformylase behaves as a monomeric species under the conditions.

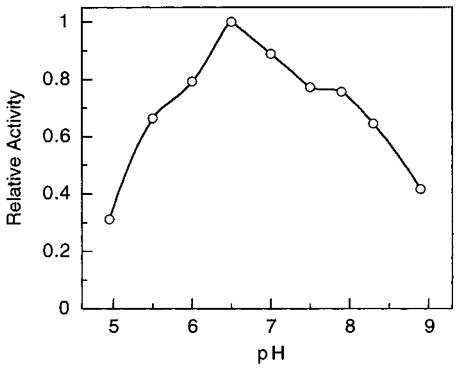


FIGURE 3: pH dependence of deformylase. The reactions were performed as described under Experimental Procedures, using 800 μM f-MAS as substrate. Activities are relative to the value at optimal pH. Multiple experiments were conducted, and data from a typical experiment are presented.

Coupled Deformylase Assay. Deformylase reactions were coupled with a formate dehydrogenase (FDH) from *Pseudomonas oxalaticus*, which oxidizes formate to CO_2 while reducing an equivalent of NAD^+ to NADH (Johnson et al., 1964). Since NADH formation is proportional to the amount of formate oxidized, production of formate by the deformylase can be monitored by following the absorbance change at 365 nm on a spectrophotometer (this wavelength is chosen to minimize the absorbance due to proteinaceous species). A standard line (OD_{365} vs formate concentration) generated using various known concentrations of sodium formate gives a slope of 2.58 $OD_{365}\cdot mM^{-1}$. The amount of formate produced by the deformylase in the coupled reactions can then be determined by comparing the absorbance at 365 nm with this standard line. The coupled reactions could in principle be monitored over time, so that initial rates could be calculated from reaction progression curves. In fact, Lazennec and Meinnel (1997) have recently reported the use of FDH from *Candida boidinii* to monitor formate release in a continuous fashion. In this work, however, we have adopted an end-point version for economical reasons. After a deformylase reaction has been terminated, FDH is added, and the formate released is quantitatively converted to CO_2. Measurement of NADH concentration at the end of the dehydrogenase reaction gives the amount of released formate. This end-point assay consumes much less FDH per experiment. This method works well with all N-formylated peptide substrates. It is very sensitive, capable of detecting formate in the low micromolar range.

pH Profile. The effect of pH on deformylase activity was assessed using the tripeptide formyl-MAS as substrate (800 μM). The enzyme displays a pH optimum near neutral pH (pH 7.0), with lower activities at either pH extreme (Figure 3). Other tested substrates such as f-MA (data not shown) and f-ML-*p*-nitroanilide (Wei & Pei, 1997) all gave qualitatively similar pH profiles. This contrasts with an earlier report by Meinnel and Blanquet (1995) that the deformylase had a broad pH optimum, with constant activity from pH 6.5 to 11.2 (assayed against f-MAS). It is likely that the different expression and purification procedures used in these two groups have resulted in some subtle differences in the protein structure.

Substrate Sequence Specificity. The catalytic properties of peptide deformylase toward a variety of formylated peptides were assessed at pH 7.0 using the dehydrogenase assay. The second-order rate constants for these peptides,

Table 1: Catalytic Constants of Peptide Deformylase[a]

substrate	k_{cat}/K_M ($\times 10^4$ $M^{-1}\cdot s^{-1}$)	substrate	k_{cat}/K_M ($\times 10^4$ $M^{-1}\cdot s^{-1}$)
f-M	0.00051 ± 0.00006	f-HASR	0.011 ± 0.002
f-MA	1.44 ± 0.02	f-(Nle)AS	2.09 ± 0.07
f-MAE	0.44 ± 0.03	f-AGSE	NDA
f-MAS	2.94 ± 0.17	f-(D-Met)AS	NDA
f-MASR	1.55 ± 0.03	f-M(D-Ala)SR	0.11 ± 0.01
f-MASDRE	7.3 ± 2.6	Ac-MAS	NDA
f-MFNR	0.66 ± 0.03	f-ML-pNA	187 ± 16
f-(Mox)FNR	NDA		

[a] All reactions were carried out in 50 mM sodium phosphate or Hepes, pH 7.0, and 10 mM NaCl at 23 °C. Abbreviations: f, formyl; A, alanine; E, glutamic acid; S, serine; R, arginine; D, aspartic acid; F, phenylalanine; N, asparagine; H, histidine; Nle, norleucine; G, glycine; Mox, methionine sulfoxide; Ac, acetyl; L, leucine; pNA, *p*-nitroanilide; NDA, no detectable activity.

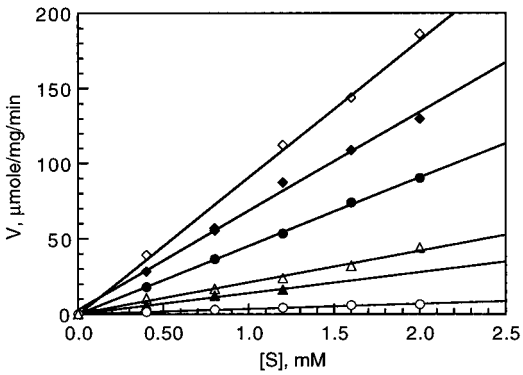


FIGURE 4: Velocity vs [substrate] plots for deformylation of various peptides. Open circles, f-M(D-Ala)SR; closed triangles, f-MAE; open triangles, f-MFNR; closed circles, f-MA; filled diamonds, f-NleAS; and open diamonds, f-MAS. Reactions were carried out in buffer D or E containing 0−2.0 mM substrate and 1.6−15 μg of deformylase. The substrate to product conversion was kept at <10% in most reactions. The curves were fitted to the data according to the equation: $V = V_{max}\cdot[S]/(K_M + [S])$. Data from a typical experiment are presented.

k_{cat}/K_M, are listed in Table 1. These data allow us to draw several conclusions. First, a prominent feature is that for most substrates tested, the enzyme shows no saturation kinetics up to ~2 mM substrate. Velocity vs substrate concentration plots typically produce a straight line, indicating that the K_M values for these substrates are >2 mM (Figure 4). The only exception so far has been the dipeptide f-ML-*p*-nitroanilide, which exhibits saturation kinetics with a K_M of 20.3 μM, a k_{cat} of 38 s^{-1}, and a k_{cat}/K_M of 1.9×10^6 M^{-1} s^{-1} (Wei & Pei, 1997). The generally high K_M values suggest that the enzyme has relatively low affinity to these small peptides. Nonetheless, the deformylase is still a rather robust catalyst ($k_{cat}/K_M = 10^4-10^6$ $M^{-1}\cdot s^{-1}$). These activities are 2−4 orders of magnitude higher than those reported earlier (e.g., $k_{cat}/K_M \sim 80$ M^{-1} s^{-1} for f-MAS) (Meinnel & Blanquet, 1995). Still higher k_{cat}/K_M values are possible if assayed against optimal substrates.

Second, the deformylase has strong sequence specificity. It exhibits strong preference for a methionine at the N-terminus of a peptide substrate, having little activity toward f-AGSE and greatly attenuated activity for f-HASR (k_{cat}/K_M = 110 $M^{-1}\cdot s^{-1}$ for f-HASR vs 1.55×10^4 $M^{-1}\cdot s^{-1}$ for f-MASR). A small modification of the methionine such as oxidation into methionine sulfoxide abolishes the deformylation process [compare f-MFNR and f-(Mox)FNR]. Norleucine is the only substitution at the N-terminus that retains most of the deformylation activity. The deformylase also has sequence selectivity at other positions, although not as strong as at the N-terminus. Among the *N*-formylmethionyl substrates tested, the k_{cat}/K_M values vary from 0.11×10^4 to 1.9×10^6 $M^{-1}\cdot s^{-1}$ (Table 1). For instance, peptides f-MAS and f-MAE are different only at the third residue and yet have a 7-fold difference in k_{cat}/K_M values. Similarly, extension of f-MAS by three residues (e.g., f-MASDRE) further increases its rate of deformylation by 2−3-fold.

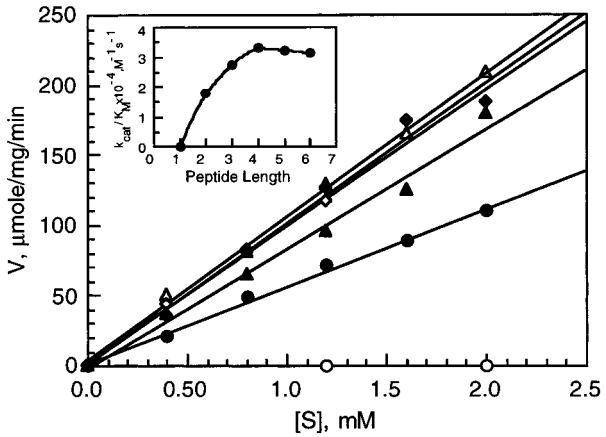


FIGURE 5: Substrate size dependence. Peptides were treated with 2.5−5.0 μg of the deformylase for 30 s at room temperature. Open circles, f-M; closed circles, f-MS; closed triangles, f-MSS; open triangles, f-MSSS; filled diamonds, f-MSSSS; and open diamonds, f-MSSSSS. The curves were fitted to the data according to the Michaelis−Menten equation. Inset: secondary plot treatment of k_{cat}/K_M as a function of peptide length.

Third, the deformylase exhibits interesting catalytic properties toward peptides containing unnatural amino acids. It shows no activity to f-(D-Met)AS, which has a D-methionine at the N-terminus, indicating that an L-amino acid at the N-terminus is essential for activity. However, deformylase can apparently tolerate a D-amino acid residue at the penultimate position and perhaps all other positions thereafter. For example, tetrapeptide f-M(D-Ala)SR is readily deformylated by the *E. coli* enzyme.

Fourth, the deformylase has strong preference for formylated vs other acylated peptides. While it efficiently cleaves the formyl group from f-MAS, it shows no detectable deacetylation of acetyl-MAS as judged by ninhydrin test (Moore & Stein, 1954). When the acetylated and trifluoroacetylated analogs of f-ML-*p*-nitroanilide (which is the best substrate identified so far) were tested, the deformylase showed a small amount of deacylation, with rates ~10^4-fold lower than that of deformylation (Wei & Pei, 1997).

Substrate Size Specificity. The size requirement of the deformylase was analyzed using a set of *N*-formylmethionyl peptides of one to six amino acids. Polyseryl peptides (f-MS_{1-5}) were used to improve their solubility and to avoid any unfavorable charge interactions. The enzyme acts as a *peptide* deformylase requiring at least a dipeptide as substrate; it deformylates formylmethionine extremely slowly (k_{cat}/K_M = 5 M^{-1} s^{-1}), consistent with the earlier reports (Adams, 1968; Meinnel & Blanquet, 1995). The deformylation rate increases with the peptide length until it reaches the tetrapeptide, where the deformylation rate reaches a plateau (Figure 5). This indicates that the substrate binding pocket of the deformylase is extended to interact with the

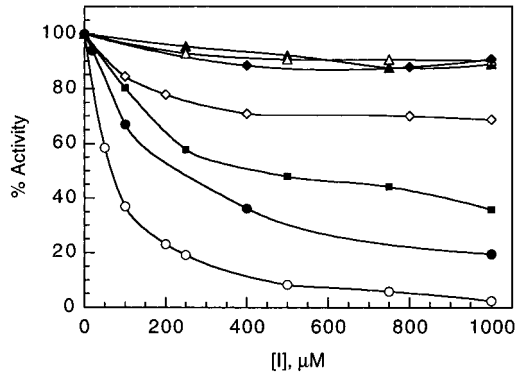


FIGURE 6: Effect of various reagents on deformylase activity. Closed triangles, iodoacetic acid; open triangles, EDTA; filled diamonds, glutathione; open diamonds, 2-mercaptoethanol; squares, thiophenol; closed circles, 1,10-phenanthroline; and open circles, *p*-(chloromercuri)benzoate. Reactions were carried out with 1.0 mM f-MA [100 μM f-ML-pNA and a continuous assay (Wei & Pei, 1997) were used for *p*-(chloromercuri)benzoate, which is also a potent inhibitor for formate dehydrogenase] as substrate for 30 s.

N-terminal four amino acids of a substrate. One thus expects that an optimal substrate for the deformylase should encompass at least four amino acid residues with a proper sequence combination.

Effect of Small Effector Molecules on Deformylase. Since peptide deformylase contains a metal ion and an essential cysteine as metal ligand (Meinnel et al., 1995; Chan et al., 1997), we tested several divalent metal-chelating agents (1,10-phenanthroline, 2-mercaptoethanol, glutathione, EDTA, and thiophenol) and thiol-specific agents [*p*-(chloromercuri)-benzoate and iodoacetate] for their effects on the deformylase. These effector molecules were directly added to deformylase reactions (reaction time 30 s), and initial rates were measured (Figure 6). Among these effector molecules, *p*-(chloromercuri)benzoate is the most potent inhibitor, with an IC_{50} of 70 μM. This inhibition is due to time-dependent inactivation of the enzyme (data not shown). Peptide deformylase has two cysteines at positions 90 and 129, respectively (Mazel et al., 1994). Cys-90 is an essential ligand for the metal (Meinnel et al., 1996; Chan et al., 1997) whereas Cys-129 can be mutated without effect on its activity (Meinnel et al., 1995). The inhibition is likely caused by slow sequestration of Cys-90 by the organomercuryl compound, although modification at Cys-129 or other residues cannot be completely ruled out as the cause of inhibition. Ligation to organomercuryls by free thiols usually takes place instantaneously. The slow inhibition kinetics are consistent with the fact that Cys-90 is ligated to the metal ion in the protein and thus not readily accessible to the mercuryl. The lack of significant inhibition by iodoacetate, a thiol-specific reagent, also supports this notion. The crystal structure shows that Cys-90 and Cys-129 are both relatively exposed to solvent (Chan et al., 1997).

Divalent metal chelators exhibit a mixed behavior. Deformylase is strongly inhibited by 1,10-phenanthroline (IC_{50} = 200 μM). The inhibition appears to be competitive with an inhibition constant (K_i) of 170 μM. EDTA showed only slight inhibition (~10% at 1 mM). Prolonged exposure to EDTA (e.g., dialysis), however, causes loss of deformylase activity, in contrast with an earlier report (Meinnel & Blanquet, 1995). The difference between phenanthroline and EDTA, both of which are excellent metal chelators, is likely due to different accessibility to the enzyme-bound metal.

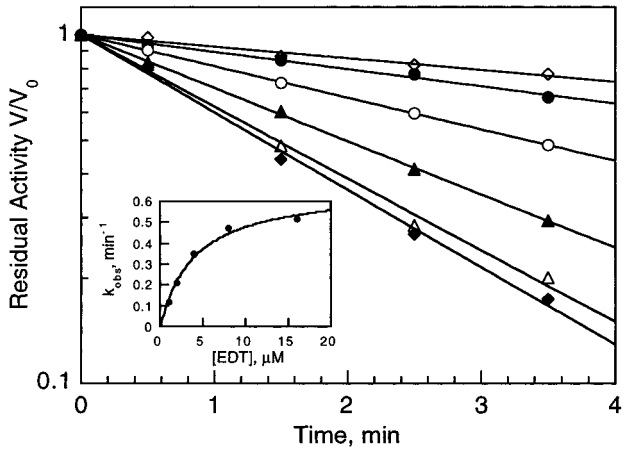
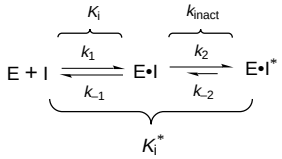


FIGURE 7: Time-dependent inhibition of peptide deformylase by ethanedithiol. Deformylase was incubated with the indicated amount of EDT at 4 °C for the indicated times. The residual activity was measured using f-ML-pNA as substrate (Wei & Pei, 1997). Open diamonds, no EDT; closed circles, 1 μM EDT; open circles, 2 μM; closed triangles, 4 μM; open triangles, 8 μM; and filled diamonds, 16 μM. Inset: secondary treatment of k_{obs} and [I] to determine K_i and k_{inact}.

Interestingly, although low-molecular-weight monothiols were reported to strongly inhibit the crude deformylase (Adams, 1968), they are only weak inhibitors for the purified enzyme, with ~30% inhibition at 1 mM 2-mercaptoethanol. Thiophenol is slightly more potent, having an IC_{50} of ~500 μM (Figure 6). Glutathione shows ~10% inhibition at 1 mM.

Time-Dependent Inhibition of Deformylase by Dithiols. Weak inhibition of the deformylase by monothiol compounds prompted us to test the effect of dithiol compounds, which are potent slow-binding inhibitors for VanX, a zinc metallopeptidase involved in vancomycin resistance in *Enterococcus faecium* (Wu & Walsh, 1996). Dithiols such as ethanedithiol, 1,3-propanedithiol, and 2,3-dimercapto-1-propanol were indeed found to be very potent inhibitors of the deformylase. Their inhibition exhibits time-dependent kinetics (Figure 7) and can be described by a slow-binding model (Morrison & Walsh, 1988):



where the deformylase forms an initial complex, E·I, in a rapid step, followed by a slow conversion of the initial complex into a more stable complex, E·I*. The potency of an inhibitor can be described by an initial inhibition constant, K_i, and an overall inhibition constant, K_i*. The rate for conversion of the initial complex into the tight complex can be described by an apparent inactivation constant, k_{inact}. Inhibition constants K_i and k_{inact} can be obtained by fitting the observed inactivation constants, k_{obs}, against the equation:

$$1/k_{obs} = (K_i/k_{inact})(1/[I]) + 1/k_{inact}$$

Because the instability of the deformylase makes it impossible to accurately determine the rate constants k_2 and k_{-2} (which requires the measurement of residual activity after prolonged incubation with inhibitor), K_i* could not be

Table 2: Kinetic Parameters of Dithiol Compounds as Time-Dependent Inhibitors[a]

inhibitor	K_i (μM)	k_{inact} (min^{-1})
1,2-ethanedithiol	4.0 ± 0.8	0.67 ± 0.05
1,3-propanedithiol	2.9 ± 0.7	0.71 ± 0.06
2,3-dimercapto-1-propanol	10.9 ± 2.0	0.52 ± 0.03
2,3-dimercapto-1-propanesulfonic acid	40.2 ± 8.8	0.82 ± 0.08
1,5-pentanedithiol	38 ± 5[b]	ND[c]

[a] All reactions were performed at 4 °C with 100 μM f-ML-pNA as substrate. [b] Estimated K_i assuming the inhibition is competitive. [c] ND, no detectable time-dependent inactivation.

determined (Morrison & Walsh, 1988). Thus, one cannot rule out the possibility of time-dependent inactivation of the deformylase (where $k_{inact} = k_2$, $k_{-2} = 0$). Plasma emission spectroscopic analysis shows that ethanedithiol-treated deformylase has the same Zn^{2+} content as the untreated protein, indicating that inhibition by dithiols is not due to Zn^{2+} removal. The inhibition constants K_i and k_{inact} for several dithiol compounds are summarized in Table 2.

Among the dithiol compounds tested, 1,3-propanedithiol and ethanedithiol are most potent, with K_i values of 2.9 μM and 4.0 μM, respectively. The rates for the conversion of E·I to E·I* (k_{inact}) are relatively fast for both molecules, at $\sim$0.7 min^{-1}. To obtain an estimate for the magnitude of k_{-2}, ethanedithiol-inhibited deformylase was quickly passed through a Pharmacia fast desalting (G-25) column to remove the excess thiols (a typical run takes $\sim$40 s with flow rate set at 6.0 mL/min). The eluted enzyme was incubated at 4 °C, and the recovered activities were measured at various times between 1 and 60 min. Less than 2% of the original activity was detected at any time point, whereas 82% activity was recovered in a control experiment in which ethanedithiol was excluded. Dialysis, dilution, or addition of organomercuryls to remove the dithiols also failed to recover the deformylase activity. These results suggest that conversion of E·I to E·I* is effectively irreversible ($k_2 \gg k_{-2}$). Since $K_i^* = K_i \cdot k_{-2}/(k_2 + k_{-2})$, the apparent inhibition constant K_i*s should be significantly lower than the corresponding K_i values, perhaps in the low nanomolar range.

Other 1,2- and 1,3-dithiol compounds tested also show time-dependent inhibition of peptide deformylase but are less potent (Table 2). Note that 2,3-dimercapto-1-propanol and 2,3-dimercapto-1-propanesulfonic acid used are racemic mixtures. No attempt has been made to test their individual stereoisomers. An interesting exception has been 1,5-pentanedithiol. It appears to act as a simple competitive inhibitor with a K_i of 38 μM. No clear time-dependent inhibition was observed at the concentrations tested (up to 90 μM).

DISCUSSION

Crude deformylase is extremely labile, and this extraordinary instability has for decades prevented its purification or any attempt of mechanistic investigation (Adams, 1968; Livingston & Leder, 1969). In this work, we have overexpressed the *E. coli* peptide deformylase and purified this enzyme to homogeneity. Although this deformylase has previously been overexpressed and purified by others (Meinnel & Blanquet, 1993, 1995), the system described here produces significantly higher yields of the purified deformylase (>50 mg from a liter of cells). More importantly, *our system has for the first time produced highly active deformylase*. We have found that the activity of purified deformylase varies drastically depending upon the purification procedure used. Meinnel and Blanquet (1993) also reported high levels of deformylase activity immediately after cell lysis, but that activity was rapidly lost during purification steps. It appears that the purification procedure employed by the other workers resulted in largely inactive proteins. The ready availability of pure, highly active deformylase has permitted us to begin a thorough examination of its catalytic properties, some of which have been found to be rather different from what was reported previously.

Deformylation *in vivo* is believed to be cotranslational (Pine, 1969), but little is known about the average length of the polypeptides when they are deformylated. Studies with a set of *N*-formylmethionylseryl peptides indicate that the enzyme active site can interact with the N-terminal four residues of a substrate. Elongation of a substrate beyond a tetrapeptide does not seem to increase the reaction rate. Thus, *in vivo* deformylation could in principle take place immediately following the translational initiation.

Peptide deformylase displays sequence specificity. It strongly prefers a methionine residue at the N-terminus. Substitution of the methionine for other amino acid residues or oxidation to methionine sulfoxide severely reduces the deformylation rate. The identity of residues after the N-terminal methionine is not so critical, as all *N*-formylmethionyl peptides tested are reasonably good substrates. This broad specificity is necessary for the enzyme to efficiently remove the formyl group from all of the newly synthesized polypeptides. However, it is also clear that the exact nature of these N-terminal residues does have some influence on the rate of deformylation (Table 1), indicating that all proteins may not be deformylated at an equal rate *in vivo*.

A small fraction of bacterial proteins are known to retain their formyl groups *in vivo* (Hauschild-Rogat, 1968; Tsunasawa et al., 1983; Flinta et al., 1986; Milligan & Koshland, 1990). Whether their folded structures affect the deformylation rate is still unknown. Our observation that tetrapeptide f-MFNR, after oxidation of its methionine side chain, becomes resistant to deformylase action may offer a plausible explanation why these proteins retain their N-terminal formyl groups. Perhaps the N-terminal methionines in these proteins are more susceptible to oxidation. This oxidation can be caused by the byproducts of aerobic metabolism such as the superoxide ion, hydrogen peroxide, and hydroxyl radical (Brot & Weissbach, 1991). We have found that simple exposure to air for several days is sufficient to completely oxidize f-MFNR to its methionine sulfoxide counterpart, while other methionyl peptides are not affected. In fact, in order to demonstrate that f-MFNR is a substrate for the deformylase, anaerobic conditions had to be used for handling the peptide. *Salmonella typhimurium* aspartate chemoreceptor, a well-known example of formyl retention *in vivo* (Milligan & Koshland, 1990), has the sequence f-MFNR at its N-terminus.

Deformylation reactions as catalyzed by the *E. coli* enzyme occur via a hydrolytic process. Both formate and a peptide with a free amino terminus are the products of the reaction (Adams, 1968). This puts the deformylase into the exopeptidase family which includes aminopeptidases and acylases. Besides the *E. coli* enzyme, the *def* genes have also been identified from *Thermus thermophilus* (Meinnel & Blanquet,

1994), *Haemophilus influenzae* (Fleischmann et al., 1995), and *Mycoplasma genitalium* (Fraser et al., 1995). The four deformylase proteins have similar sizes (169−226 amino acids) and show extensive sequence homology (28−65% identity). They share no overall sequence homology to any other known proteins (Mazel et al., 1994), suggesting that the deformylases may form their own protein family. Indeed, the three-dimensional structure as determined by NMR (Meinnel et al., 1996) and X-ray diffraction (Chan et al., 1997) shows a distinct structural fold. The only discernible similarity to other proteins is the presence of a conserved sequence motif, HEXXH (X, any amino acid), which is frequently found in zinc metallopeptidases where it is used to provide two ligands (the two histidines) to the zinc metal (Vallee & Auld, 1990). The deformylase indeed contains a metal ion, ligated by the two histidine residues (Meinnel et al., 1996; Chan et al., 1997). Meinnel and co-workers (Meinnel & Blanquet, 1993, 1995; Meinnel et al., 1995, 1996) have proposed that this metal is Zn^{2+}. A surprising finding from the structural work is that Cys-90 acts as the third ligand for the metal, whose role appears to be catalytic (*vide infra*). Although over 300 zinc-containing enzymes and proteins have been identified (Vallee & Auld, 1990), there have been only a handful of proteins which utilizes cysteine(s) as a ligand(s) for a catalytic zinc (alcohol dehydrogenases and cytidine deaminase). None of these zinc enzymes with cysteine ligands are amide hydrolases. If zinc is the catalytic metal, peptide deformylase would represent a new subfamily of amide hydrolyases. Our active deformylase does contain a Zn^{2+} ion, but also some other metals at significant levels (Fe^{2+} or Fe^{3+}, Cu^{2+}, Co^{2+}). Thus, while Zn^{2+} is likely the native metal of peptide deformylase, one cannot rule out the possibility of other metals playing the catalytic role. Experiments are being carried out in this laboratory to establish the identity of the metal in the *active* deformylase.

Crude deformylase was found to be sensitive to low-molecular-weight thiols (Adams, 1968). The purified enzyme is weakly inhibited by monothiols but strongly inhibited by dithiols. We and others (Meinnel & Blanquet, 1995) have found that divalent metal chelators such as 1,10-phenanthroline also inhibit the deformylase ($K_i = 170\ \mu M$), apparently by forming a ternary complex involving the enzyme, the metal, and the inhibitor. It is likely that these sulfhydryl compounds and 1,10-phenanthroline inhibit the deformylase by directly binding to the metal ion. Although other possibilities (e.g., conformational changes) cannot be completely ruled out, these results suggest that the metal ion is located in the active site of deformylase and participates directly in catalysis. The 3-D structure reveals that the metal ion sits at the bottom of a cleft, the putative active site (Meinnel et al., 1996; Chan et al., 1997). The sides of the cleft are formed by two conserved loops (amino acids 40−48 and 89−96). Titration with substrate analogs shifted the ^1H to ^{15}N correlation peaks for several residues surrounding the metal-binding site (Meinnel et al., 1996). The metal ion could conceivably coordinate with the carbonyl oxygen of the formyl group, polarize the carbonyl group, and thus facilitates the nucleophilic attack by a water molecule, as proposed for carboxypeptidase A (Vallee et al., 1983). It is also conceivable that the metal ion binds and ionizes a nucleophilic water molecule and the resulting metal-bound hydroxide ion attacks the formyl carbonyl, as discussed in the preceding paper (Chan et al., 1997). In either mechanism, binding of these inhibitors to the metal ion would prevent a substrate from approaching the active site.

As for the time-dependent inhibition by the dithiol compounds, we propose the following mechanism. The first thiol group displaces the fourth ligand of the tetrahedral metal ion, a bound water molecule, in a rapid step. During the slow conversion to a more stable complex, the second thiol group on the dithiol compounds ligates to the metal ion to form a very stable, bidentate five- (for 1,2-dithiols) or six-membered (for 1,3-dithiols) ring structure that mimics the transition state of the deformylase-catalyzed reaction. This conversion may require some substantial changes in the protein structure, making the process relatively slow. One possible structural change may be the dissociation of one of the protein ligands to form a new tetracoordinated metal complex, with two ligands coming from the protein and two ligands from the inhibitor. Since the dithiols failed to remove the Zn^{2+} ion, this mechanism is likely if Zn^{2+} is the catalytic metal. Alternatively, the structural change could be due to removal of the metal ion from the protein. This mechanism cannot be true if Zn^{2+} is the metal but may be operative if other divalent metal ions are the native metal of peptide deformylase. The lack of time-dependent inhibition by 1,5-pentanedithiol supports both models. Bidentate coordination with a metal ion by 1,5-pentanedithiol would require the formation of a highly strained eight-membered ring, which is energetically disfavored. As a result, 1,5-dithiols behave like the monothiol compounds, only capable of forming the initial complex, E·I. Structural characterization of the enzyme/inhibitor complex is already under way.

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