Peptide deformylase catalyzes the removal of the N-formyl group from newly synthesized polypeptides in prokaryotes. Its essential character and unique presence in prokaryotes make it an attractive target for antibacterial chemotherapy. However, purification and characterization of the peptide deformylase have remained a major challenge because this enzyme is extraordinarily labile under a variety of conditions (1/2min at room temperature). In this work, we show that this unusual instability is because of oxidation of the catalytic Fe$^{2+}$ ion of the deformylase into catalytically inactive Fe$^{3+}$ ion by atmospheric oxygen. Oxidation of Fe$^{2+}$ is accompanied by the conversion of O$_2$ into a yet unidentified reactive species, which covalently modifies the deformylase protein, most likely by oxidizing cysteine-90, a ligand residue of the Fe$^{2+}$ ion, into a cysteine sulfonic acid. Enzymatic exclusion of O$_2$ from the deformylase renders the deformylase highly stable under otherwise identical conditions. An improved, readily reproducible purification procedure has been developed that produces approximately 10 mg of pure, fully active Fe$^{2+}$ deformylase from a liter of cells. In addition, active peptide deformylase can be reconstituted in vitro from the denatured deformylase.

Protein synthesis in prokaryotes initiates with an N-formylmethionyl-tRNA, resulting in N-terminal formylation of all nascent polypeptides (1). Peptide deformylase catalyzes the subsequent removal of the formyl group from the majority of bacterial proteins (2–4). Although the precise functions of the formylation and deformylation steps remain somewhat obscure, genetic studies have shown that the peptide deformylase activity is essential for bacterial survival (5, 6). Because peptide deformylase is apparently absent in eukaryotic systems (7, 8), it provides a potential target for a novel antibacterial strategy (5).

Very recently, biochemical studies from this laboratory have established that peptide deformylase represents an intriguing new class of amide hydrolyase, which utilizes a ferrous ion as the catalytic metal (7). The unusual structural feature of peptide deformylase and its potential application in antibiotic studies warrant a detailed mechanistic investigation of this class of enzymes.

Although the deformylase activity was recognized three decades ago (2–4), the extraordinary lability of this enzyme has long prevented its purification and characterization. It was reported that even mild procedures such as dialysis, Sephadex G-100 or DEAE-cellulose chromatography, or ultracentrifugation on sucrose gradient resulted in almost quantitative loss of enzymatic activity (2, 3). At 37 °C, the deformylase in a crude cell lysate lost activity with a half-life of 60 s (3). We have recently overexpressed the deformylase from Escherichia coli and purified the active enzyme to homogeneity (7, 9). Unfortunately, the purified enzyme remains highly unstable, complicating the quantitative evaluation of its activity. In this work, we have found that the instability of peptide deformylase is because of oxidation of the catalytic Fe$^{2+}$ ion into the catalytically inactive ferric ion by atmospheric O$_2$. A simple experimental procedure has been developed to preserve the deformylase activity. These findings will greatly facilitate future mechanistic investigations of the peptide deformylase as well as high throughput screening for deformylase inhibitors.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glucose oxidase (EC 1.1.3.4.), superoxide dismutase, and catalase (EC 1.11.1.6) from Aspergillus niger were purchased from Fluka (Ronkonkoma, NY). Acromonas aminopeptidase and all chemicals were from Sigma Chemical Company.

**Buffers**—All purification buffers were exhaustively degassed under vacuum and then sparged with argon prior to use. Buffer A, 20 mM potassium phosphate, pH 8.0, 10 mM NaCl, 1% Triton X-100, 0.5% protease inhibitor mixture (100 μg of phenylmethylsulfonyl fluoride/ml, 20 μg trypsin inhibitor/ml, 20 μg leupeptin/ml, 20 μg pepstatin/ml). The cells were lysed by incubation for 30 min at 4 °C followed by sonication (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 was loaded onto a Q-Sepharose Fast Flow (Amerham Pharmacia Biotech) column (2.5 × 7.5 cm) that had been 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 4 ml/min. The deformylase eluted at approximately 100–220 mM NaCl. Active fractions were pooled (~50 ml) and precipitated by adjusting to 80% saturation with ammonium sulfate. The precipitate was redissolved in ~12 ml of buffer C plus 1.2 mM ammonium sulfate and loaded onto a Pharmacia HiLoad 16/10 phenyl-Sepharose column that had been equilibrated in buffer C plus 1.7 mM ammonium sulfate. Elution was performed with buffer C and a reverse ammonium sulfate gradient (1.7–0 M; 200 ml at 4 ml/min). The deformylase was separated into two species on this column, with peak 1 (eluted at ~1.0 mM ammonium sulfate) being the Zn$^{2+}$-containing enzyme and peak 2 (eluted at ~0.76 mM ammonium sulfate) being the Fe$^{2+}$-containing enzyme. The active fractions under each peak were pooled separately and concentrated in a Centricon-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 by the method of Bradford (10) using BSA as standard and then corrected according to
the equation: actual concentration = 0.71 x concentration from Bradford (based on quantitative metal analysis of pure Fe²⁺ and Zn²⁺ deformylases).

**Determination of Fe⁴⁺ and Fe³⁺**—The Fe³⁺ and Fe⁴⁺ contents in deformylase were measured by modification of a literature procedure (12). To measure Fe³⁺ content, the deformylase protein (typically 2–4 mg/ml) was denatured by the addition of an equal volume of 8 M GnHCl, and 1,10-phenanthroline in 0.5 M sodium acetate (pH 5.0) was immediately added to a final concentration of 5–10 mM. The absorbance at 510 nm was measured on the **Aeromonas** albumin; ESI, electrospray ionization; MS, mass spectrometry; HPLC, high pressure liquid chromatography.

**Determination of Fe⁴⁺ and Fe⁵⁺**—The Fe⁴⁺ and Fe⁵⁺ contents in deformylase were measured by modification of a literature procedure (12). To measure Fe⁴⁺ content, the deformylase protein (typically 2–4 mg/ml) was denatured by the addition of an equal volume of 8 M GnHCl, and 1,10-phenanthroline in 0.5 M sodium acetate (pH 5.0) was immediately added to a final concentration of 5–10 mM. The absorbance at 510 nm was measured on the **Aeromonas** UV-visible spectrophotometer at 405 nm. The initial rate was calculated over the first 30 s. Under these conditions, the deformylase is saturated with the substrate and therefore, the maximum velocity (Vₘ) was measured.

**Activity and Fe⁵⁺ Content**—Freshly thawed, highly active deformylase in buffer D (2.8 mg of enzyme in 1 ml) was incubated at room temperature, exposed to atmospheric oxygen. At various times (0–26 h), 50-μl aliquots were withdrawn and the remaining catalytic activity and Fe⁵⁺ content were determined as described above. The Fe⁴⁺ and Fe⁵⁺ contents were also determined at the conclusion of the experiment.

**Liquid Chromatography ESI-MS Analysis**—Salt free deformylase samples (typically 10–50 μg) were dissolved in 0.1% trifluoroacetic acid and passed through a C-8 reversed-phase HPLC column (eluted with acetonitrile in 0.1% trifluoroacetic acid) before being injected into a Perkin-Elmer mass spectrometer (model API-300). To locate the site(s) of modification in the deformylase, the highly active Fe⁴⁺ deformylase (1 mg/ml) was exposed to air for 14 h at room temperature; this procedure resulted in loss of >99% deformylase activity. The inactivated enzyme was then digested to completion with 0.1 molar equivalent of trypsin in 100 mM Tris-HCl, pH 8.0 (12 h at room temperature). The digest was passed through a C-18 reversed-phase column, eluted with a linear gradient of acetonitrile in deionized H₂O containing 0.1% trifluoroacetic acid (0–100% acetonitrile in 30 min at 1 ml/min) before mass spectrometric analysis. All major peaks in the mass spectra were identified by comparison with a tryptic map of the deformylase using Bio MultiView 1.2 software (Perkin-Elmer Inc.).

**Low Oxygen Environment**—The glucose oxidase and catalase system was chosen to generate and maintain a partially anaerobic environment (13). A solution (1.0 ml) containing buffer D, 100 μM FMN-pNA, 0.4 units of **Aeromonas** aminopeptidase, 20 mM D-glucose, 0.5 units of glucose oxidase, and 700 units of catalase was prepared in a polystyrene cuvette. The contents were mixed, and 0.5 ml of mineral oil was immediately added to form a thin layer on top of the aqueous solution, which helps to deter atmospheric O₂ from entering the assay solution. The mixture was incubated at room temperature for 2–5 min, and the reaction was initiated by the addition of 50 ng of the deformylase, which was carried out by inserting a pipette tip through the mineral oil layer into the aqueous phase.

**Preparation of Metal-free Deformylase—Zn²⁺ deformylase** (4.5 mg/ml), previously referred to as peak 1 (7), was denatured by the addition of an equal volume of 8 M GnHCl. EDTA was then added to the final concentration of 100 mM. The resulting mixture was applied to a Pharmacia Superdex 75 column equilibrated in 4 M GnHCl, and the column was eluted with 4 M GnHCl. Fractions containing the deformylase (collected at 280 nm) were collected and concentrated in a Centricon-10 apparatus before its use in reconstitution experiments.

**Refolding of Deformylase**—Refolding experiments were carried out directly under the assay conditions described above but in the presence of 100 μM Zn²⁺, Fe²⁺, or Fe³⁺. Typically, 1.5 μg of metal-free deformylase (10 μl) in 4 mM GnHCl was added to 1.0 ml of buffer D containing 100 μM FMN-pNA, 0.4 unit **Aeromonas** aminopeptidase, and 100 μM metal ion. Refolding was monitored by following the hydrolysis of FMN-pNA spectrophotometrically at 405 nm (11). Control experiments without metal ion were also performed. Refolding in a low oxygen environment was similarly carried out but in the presence of glucose, glucose oxidase, and catalase.

**RESULTS AND DISCUSSION**

Extraordinary lability has been a hallmark of peptide deformylase. Although high levels of deformylase activity were readily detectable in crude, freshly prepared cell lysates, this activity was rapidly lost (t₁/₂ ~ 1 min) upon any attempt of purification (e.g. gel filtration or ion exchange chromatography, sucrose sedimentation) (2–4). These observations led to earlier proposals that peptide deformylase requires cofactor(s) that are removed by the purification procedures. We have recently succeeded in the purification of *E. coli* deformylase in large quantities through overexpression (7, 9). The extensively purified deformylase (the Fe²⁺-containing form) is catalytically active but remains highly unstable. When incubated at room temperature under deformylase assay conditions, where the enzyme is usually present at low concentrations (~0.1 μg/ml), it is inactivated at a half-life of ~1 min. The enzyme is considerably more stable at 4 °C, but still loses ~40% activity in 10 min. The deformylase shows improved stability if stored as concentrated solutions (>1 mg/ml). However, with the addition of a carrier protein (e.g. BSA), glycerol, or a variety of common enzyme cofactors, all failed to preserve the activity. In contrast to the Fe²⁺ deformylase, the apparently nonphysiological less active variant, Zn²⁺-containing deformylase (previously described as peak 1) (7), is very stable under all of the conditions tested. These observations suggest that enzyme inactivation is likely because of factors other than loss of cofactor(s).

Because peptide deformylase is an important enzyme both from a mechanistic viewpoint (a novel Fe²⁺-containing amide) and as a potential target for antibiotic design, we have undertaken a series of biochemical investigations to determine the mechanism of its rapid inactivation. The dramatically different stability between the Fe²⁺ and Zn²⁺ deformylases, which share the same polypeptide sequence (7), prompted us to examine the valence state of iron in the inactivated deformylase, as the Fe²⁺ ion could be oxidized into the Fe³⁺ ion by atmospheric O₂. We have found that simple exposure of the active Fe²⁺ deformylase (2.8 mg/ml) to air for two days at room temperature results in complete loss of deformylase activity. This inactivation is accompanied by precipitation of the deformylase protein. Metal analysis of the inactivated sample revealed the presence of 0.75 ± 0.24 g-atoms of Fe²⁺ per polypeptide protein but no detectable Fe³⁺, indicating a quantitative conversion of Fe²⁺ into Fe³⁺ (15). Next, the kinetics of enzyme inactivation and Fe²⁺ oxidation were examined by withdrawing aliquots of a deformylase sample (2.8 mg/ml) after various times of exposure to air and simultaneously assaying for both remaining deformylase activity and Fe²⁺ content as described under “Experimental Procedures.” The time courses of the two events are superimposed (Fig. 1). Moreover, there is a linear correlation between the remaining activity and Fe²⁺ content (Fig. 1, inset). This strongly suggests that Fe²⁺ cannot replace Fe³⁺ as the catalytic metal, and oxidation of the Fe²⁺ ion to Fe³⁺, presumably by atmospheric O₂, causes deformation inactivation. Note that the half-life of the deformylase here is ~8 h, instead of 1 min as described earlier. This is because of the much higher concentration of deformylase (2.8 mg/ml versus 0.1 μg/ml used in this experiment (necessary for accurate measurement of Fe²⁺ content). As mentioned earlier, for reasons that are not yet completely understood, the deformylase is
versus time.

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lar mass analysis in the negative ion mode detected a peak with a molecular mass 1852.4 Da if the peptide carries two negative charges. Unfortunately, peptide sequencing by tandem MS/MS experiments was not successful, so the modified amino acid(s) has not been unambiguous identi-

fied. However, inspection of the peptide sequence suggests that Cys-90 is most likely the site of covalent modification. Its oxidation into a cysteic acid (cysteic sulfonic acid or sulfinic acid has previously been ob-

served in a serine/threonine phosphatase (14), a nitrile hyd-

dratase (15), and the reaction catalyzed by cysteine dioxygen-

ase (16). All three enzymes are iron metalloenzymes.

The occurrence of two modification events (Fe$^{2+}$ and Cys-90 oxidation) raises the issue of which event is causative of de-

formylase inactivation. To address this question, we carried out experiments with varying times of air exposure to examine the existence of any correlation between activity loss and cysteic acid formation. No such correlation was found despite repeated experiments. There were samples that had lost >99% deformylase activity but produced the [M + 48] peak at barely detectable levels. In fact, formation of cysteic acid has not exceeded 50% for any sample so far tested. This suggests that oxidation of Cys-90 may only be a consequence rather than the cause of deformylase inactivation.

Because both oxidation events require an oxidizing agent and the most likely candidate is atmospheric O$_2$, we examined the stability of the deformylase in a reduced O$_2$ environment. A

much more stable when it is in a concentrated form. One possibility may be that high concentrations promote protein-protein interactions and prevent O$_2$ from access to the active site. These interactions must be specific, as BSA failed to preserve the deformylase activity.

We next examined the possibility of polypeptide modification during enzyme inactivation using electrospray ionization mass spectrometry. Freshly thawed, active deformylase (both Fe$^{2+}$ and Zn$^{2+}$ forms) gave a molecular mass of 19,199 ± 2 Da (Fig. 2a), in good agreement with the calculated molecular mass of 19,197 Da (minus the N-terminal methionine). However, after the active Fe$^{2+}$ deformylase was incubated overnight at room temperature with the solution exposed to air, the resulting sample (which lost >99% activity) gave two peaks of approximately equal intensities at molecular masses of 19,199 and 19,247 Da, respectively (Fig. 2b). The appearance of the [M + 48] peak indicates that deformylase inactivation is accompa-

nied by covalent modification of the polypeptide component in addition to the oxidation of Fe$^{2+}$ to Fe$^{3+}$. To locate the site(s) of modification, the inactivated deformylase was digested with trypsin to completion and analyzed by HPLC/ESI mass spectrometry (Fig. 2c). Analysis of the entire HPLC chromatogram revealed a peptide fragment with a m/z value of 927.2 (positive ion), which does not correspond to any of the fragments derived from the native deformylase. Assuming that this peptide carries two positive charges, a molecular mass of 1852.4 Da can be calculated. This matches a trypsin fragment derived from Ser-81 to Arg-97, SGETGIEEGCLSIPEQR (calculated molecular weight = 1804.4), plus a 48-Da modification. HPLC/ESI mass analysis in the negative ion mode detected a peak with a m/z ratio of 925.2 (data not shown); this would also give a molecular mass of 1852.4 Da if the peptide carries two negative charges. Unfortunately, peptide sequencing by tandem MS/MS experiments was not successful, so the modified amino acid(s) has not been unambiguously identified. However, inspection of the peptide sequence suggests that Cys-90 is most likely the site of covalent modification. Its oxidation into a cysteic acid (cysteic sulfonic acid) would result in an addition of three oxygen atoms to the peptide, which in turn would increase the molecular mass of the peptide by 48 Da. Cys-90 is likely a ligand of the catalytic Fe$^{2+}$ metal (see below). Presumably, during the process of Fe$^{2+}$ oxidation to Fe$^{3+}$, an O$_2$ molecule is reduced to a superoxide ion or other reactive species, which might oxidatively attack the ligating cysteine (see below). The other amino acid residues in the peptide are either farther away from the iron center and/or more stable against oxidative damage and are thus less likely to be modified. The Zn$^{2+}$-containing deformylase did not produce a detectable amount of the [M + 48] peak after exposure to air for extended periods of time. Furthermore, the peptide containing the other cysteine (Cys-129) was not modified during the enzyme inactivation process, as judged by mass spectrometry (data not shown). This indicates that modification of Cys-90 is not because of nonspecific oxidation by atmospheric O$_2$. Oxidation of a cysteine to cysteic sulfonic acid or sulfinic acid has previously been observed in a serine/threonine phosphatase (14), a nitrile hyd-

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Because both oxidation events require an oxidizing agent and the most likely candidate is atmospheric O$_2$, we examined the stability of the deformylase in a reduced O$_2$ environment. A
glucose oxidase/catalase system (13) was adapted to generate and maintain a partially anaerobic environment (see "Experimental Procedures"). Indeed, when the O₂ level in the assay solution was enzymatically reduced to a minimum, peptide deformylase (0.05 μM/ml) showed remarkably increased stability with only a slight loss of activity after incubation for 10 min at room temperature (Fig. 3). In contrast, in a control experiment in which no glucose oxidase/catalase was added, the deformylase was completely inactivated after 10 min. Note that the addition of glucose/glucose oxidase/catalase resulted in slight reduction in the initial rate. This is most likely because of the introduction of a small amount of O₂ during the course of deformylase addition and its subsequent conversion by the glucose oxidase into H₂O₂, which instantaneously inactivated a small percentage of the added deformylase. Hydrogen peroxide, at micromolar levels (e.g. 100 μM), is capable of rapid and complete inactivation of the Fe²⁺ deformylase by oxidizing the Fe²⁺ ion into Fe³⁺ ion (7) and promotes the formation of the [M + 48] species and a small amount of higher molecular weight species (data not shown). Addition of either glucose oxidase/ catalase or 100 μM H₂O₂ to the Zn²⁺ deformylase had no effect on its catalytic activity or stability.

A variety of O₂ scavengers were then added to the deformylase solutions in an attempt to stabilize the enzymatic activity. Simple addition of reducing agents either had no effect or more often accelerated the enzyme inactivation (e.g. ascorbate, sodium dithionite). On the other hand, addition of these reducing agents to the O₂-depleted assay reactions had no effect in all cases. Presumably, the reducing agents convert O₂ into some reactive oxygen species (e.g. H₂O₂) (17), which in turn oxidizes the ferrous ion in the enzyme. Among the redox enzymes tested, addition of catalase significantly reduces the inactivation rate but does not completely protect the deformylase from oxidative damages. This is evident from the observation that the addition of 10, 100, or 780 units of catalase resulted in similar levels of stabilization (Fig. 4). ESI-MS analysis of the inactivated deformylase revealed that the presence of catalase reduced the relative intensity of the [M + 48] peak (data not shown). Superoxide dismutase or BSA had no effect on the enzyme.

Because the formation of Fe³⁺ deformylase during the enzyme inactivation process is complicated with concomitant modification of the polypeptide, we sought to obtain pure Fe³⁺ deformylase (without Cys-90 oxidation) through in vitro reconstitution, to determine whether Fe²⁺ to Fe³⁺ oxidation is sufficient to completely inactivate the deformylase. The less active Zn²⁺ deformylase was denatured with GnHCl in the presence of 100 mM EDTA and passed through a gel-filtration column. The resulting apoenzyme (1.5 μg/ml in 10 μl of GnHCl) was directly added to deformylase assays to initiate the reactions (reaction volume 1.0 ml), and the amount of reconstituted activities were determined by a continuous assay (11). In control experiments, addition of 100 μM Zn²⁺ and Fe³⁺ to the reactions resulted in specific activities of 0.40 ± 0.04 and 1.83 ± 0.23 μmol/mg⁻¹·min⁻¹ (based on the amount of added apoenzyme), representing 51% and 1% recovery of activity for Zn²⁺ and Fe³⁺ deformylases, respectively (Fig. 5). Reconstitution with Fe³⁺ under the partially anaerobic condition recovered 2.8% activity or a specific activity at 5.03 ± 0.57 μmol/mg⁻¹·min⁻¹. The slightly upward curvature of the reaction progression curve is consistent with a somewhat slow refolding process in the presence of Fe³⁺ (Fig. 5). This implies that in the presence of O₂, the deformylase is inactivated as it is folded, giving rise to the lower activity (as compared with that from the anaerobic experiments). All refolding experiments with 100 μM Fe³⁺ failed to achieve activities higher than the control in which no metal ion was added. Large-scale reconstitution experiments with Fe³⁺ yielded a small amount of a soluble protein but apparently without any bound Fe³⁺ ion (estimated by complex formation with SCN⁻). This indicates that Fe³⁺ deformylase either cannot be formed at all or is catalytically inactive when formed. Thus, oxidation of Fe²⁺ to Fe³⁺ is sufficient for deformylase inactivation. We were also able to recover ~50% deformylase activity (relative to the activities reconstituted from GnHCl-denatured Zn²⁺ deformylase) from a sample that had previously been completely inactivated by exposure to air, by refolding it in the presence of Fe³⁺ or Co²⁺. This is consistent with the observation that only <50% of the deformylase undergoes cysteine to cysteic acid oxidation. It also rules out cysteic acid formation as the direct cause of enzyme inactivation.

Based on the above results, we propose the following mechanism to account for the rapid inactivation of Fe³⁺ deformylase (Fig. 6). We believe that the Fe³⁺ enzyme has a similar structure to that of the Zn²⁺ variant, the structure of which has recently been solved (18, 19). Our laboratories and others² have recently solved the structures of the Co²⁺- and Ni²⁺-substituted deformylases (20), which retain almost full deformylase activity. All three structures are essentially identical, with the

² P. T. R. Rajagopalan and D. Pei, unpublished results.
the metal ion plays both a catalytic role and a structural role (18–20), loss of the metal ion would lead to unfolding of the protein. Indeed, the inactivation process is accompanied by deformylase precipitation out of solution, whereas the Zn$^{2+}$ variant is highly stable against unfolding. The fact that peptide deformylase becomes very stable upon enzymatic removal of O$_2$ from assay solutions provides strong support to this mechanism. The remarkable stability of the Zn$^{2+}$ deformylase also rules out any possibility of direct modification of the polypeptide component as the cause of activity loss.

Our earlier purification procedure produced large amounts of highly active deformylase, but the specific activity of the purified enzyme from different preparations still varied by an order of magnitude (9). Our understanding of the inactivation mechanism has permitted us to optimize the purification procedure for the Fe$^{2+}$ deformylase. Because inactivation of the deformylase is caused by O$_2$ dissolved in the buffers, the improved procedure as described under “Experimental Procedures” has eliminated the ammonium sulfate fractionation and the time-consuming gel-filtration steps. This significantly shortens the overall purification procedure, thus minimizing the exposure of peptide deformylase to O$_2$. It also allows for baseline resolution of the two enzyme forms (Fe$^{2+}$ versus Zn$^{2+}$) during the last (phenyl-Sepharose) chromatographic step. These modifications do not, however, compromise the purity of the final protein as judged by SDS-polyacrylamide gel electrophoresis analysis (data not shown). This procedure is highly reproducible and produces $\sim$10 mg of Fe$^{2+}$ deformylase with consistently high specific activity ($k_{cat}/K_m = 2.9 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$) toward fML-pNA from a liter of cells.

The difference between the results obtained in this laboratory and those reported by others is worthy of some comment. In particular, how can we purify the active deformylase, which has a $t_{1/2}$ of $\sim$1 min at room temperature, whereas the same chromatographic techniques have failed in other laboratories? Two factors are likely to have contributed to our success. First, because O$_2$ is the cause of inactivation, the buffers used in this laboratory for deformylase purification were exhaustively degassed and sparged with argon or nitrogen before use, whereas there was no mention of such procedures in other published works (2, 3, 24). Second, our overexpression system produces very high levels of peptide deformylase, approximately 50% of total soluble proteins (9). Because the deformylase is more stable as a concentrated solution (Ref. 2 and this study), the improved purification procedure as described under “Experimental Procedures” has made it possible to purify the active deformylase enzyme. Meinnel and Blanquet (24) also reported high levels of deformylase activity immediately after lysis of the non-overproducing strains, where deformylase is present at lower expression levels. In particular, how can we purify the active deformylase, which has a $t_{1/2}$ of $\sim$1 min at room temperature, whereas the same chromatographic techniques have failed in other laboratories? Two factors are likely to have contributed to our success. First, because O$_2$ is the cause of inactivation, the buffers used in this laboratory for deformylase purification were exhaustively degassed and sparged with argon or nitrogen before use, whereas there was no mention of such procedures in other published works (2, 3, 24). Second, our overexpression system produces very high levels of peptide deformylase, approximately 50% of total soluble proteins (9). Because the deformylase is more stable as a concentrated solution (Ref. 2 and this study), the high expression level in our system ensured a relatively concentrated deformylase solution throughout all stages of purification, thus significantly slowing down the inactivation kinetics. Such a procedure, however, would probably not be adequate for the purification of the native deformylase from non-overproducing strains, where deformylase is present at much lower levels. Even a small amount of lingering O$_2$ or air leakage in the system would be sufficient to inactivate all of the deformylase enzyme. Meinnel and Blanquet (24) also reported high levels of deformylase activity immediately after lysis of their overproducing cells, but this activity was quickly lost during purification. We believe that, like our results, their overexpression system also produced a mixture of Fe$^{2+}$ and Zn$^{2+}$ deformylase forms, but the Fe$^{2+}$ enzyme was rapidly inactivated by O$_2$ (the reported procedure contained long dialysis steps in apparently non-degassed solutions) and precipitated out of solution. What was left in solution was the more stable but much less active Zn$^{2+}$ deformylase.

In conclusion, we have elucidated the molecular mechanism behind the extraordinary lability of peptide deformylase, a property that has prevented mechanistic investigation for three decades. Inactivation is caused by O$_2$, which binds to the...
catalytic Fe$^{2+}$ and oxidizes it into Fe$^{3+}$, followed by Fe$^{3+}$ dissociation and protein denaturation. A simple procedure has been developed to preserve the deformylase activity by enzymatic removal of O$_2$ from assay solutions. These results will greatly facilitate future biochemical studies of peptide deformylase.

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