Peptide Deformylase: A New Type of Mononuclear Iron Protein

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The emergence of drug resistance among pathogenic bacteria has stimulated the search for novel drug targets as well as the development of new drugs that inhibit the traditional targets (e.g., ribosome and enzymes for cell wall synthesis).[1] Peptide deformylase, which catalyzes the removal of an N-terminal formyl group from newly synthesized polypeptides, appears to be ideally suited for such an exploitation. While N-terminal formylation and subsequent deformylation are a universally conserved feature of prokaryotic protein synthesis[2] and peptide deformylase is essential for bacterial survival,[3] the deformylase is apparently absent in higher animals.[4] Thus, specific deformylase inhibitors may provide a new generation of antibacterial agents. Unfortunately, although the deformylase activity was recognized three decades ago,[5] the extraordinary lability of the enzyme has prevented any purification or characterization until very recently when we[6] and others[7] overexpressed the *Escherichia coli* deformylase and purified the recombinant protein to homogeneity. Surprisingly, the recombinant enzymes obtained in the two laboratories exhibit dramatic differences in specific activity and other catalytic properties.[6] We now report that peptide deformylase is a novel mononuclear iron protein and provide a possible explanation for the conflicting results in the literature.

Peptide deformylase was previously reported to be a zinc metalloenzyme, containing one Zn^{2+} ion per polypeptide chain.[7] Sequence alignment of peptide deformylases from nine bacterial organisms revealed a highly conserved sequence motif, HEXXH (H, histidine; E, glutamate; X, any amino acid),[8] which is the signature motif of zinc metallopeptidases.[9] Site-directed mutagenesis[10] and structural determination by X-ray crystallography[11] and nuclear magnetic resonance spectroscopy[12] have established that the zinc metal is tetrahedrally coordinated by the two histidines from the signature motif, cysteine 90, and a water molecule. However, the zinc protein is a very stable but sluggish enzyme ($k_{cat}/K_M \approx 80\ M^{-1}\ s^{-1}$ with formyl-Met-Ala-Ser as substrate).[7] On the other hand, the deformylase purified in this laboratory has much higher catalytic activity ($k_{cat}/K_M = 2.9 \times 10^4\ M^{-1}\ s^{-1}$ with the above substrate) but remains highly unstable.[6] Furthermore, metal analysis[13] of our protein revealed the presence of significant amounts of iron and copper, and small amounts of calcium, cobalt, and magnesium as well as zinc. This raises the possibility that a different enzyme isoform and/or a metal ion other than zinc is responsible for the higher activity of our enzyme.

Using an improved gradient, we were able to separate our deformylase preparation into two species, referred to as peak 1 and peak 2 hereafter, on a Phenyl-Sepharose column (Figure 1a). On SDS−PAGE gels, both species migrate as a 20 kDa polypeptide (data not shown). Electrospray ionization mass spectrometric analysis[14] of acid-denatured samples gave a molecular weight of 19 198 ± 2 for both peak 1 and peak 2 enzymes (Figure 1b), consistent with the calculated molecular weight of 19 197 (minus the N-terminal methionine). These results suggest that peak 1 and peak 2 are likely to have the same polypeptide sequence.[3] Circular dichroism (CD) spectra of peak 1 and peak 2 are essentially superimposed, except for slightly more negative ellipticity in the 205−217 nm region for the peak 2 protein (Figure 1c). This indicates the presence of highly similar secondary structures and likely a similar tertiary structure for both species. However, there also exist some significant differences between the two species. First, while the peak 1 protein always contains approximately 1.0 Zn^{2+} per polypeptide, the peak 2 protein contains mostly iron (up to 1.0 iron atom per polypeptide), along with small amounts of other adventitious metals, although the iron content (and activity) varied from one preparation to another. Second, the two enzyme forms have different catalytic activities. The peak 1 enzyme has a k_{cat}/K_M value of $2.5 \times 10^4\ M^{-1}\ s^{-1}$ toward formyl-Met-Leu-*p*-nitroanilide[15] and activities toward short formylated peptides (e.g., formyl-Met-Ala-Ser) comparable to those reported previously.[7] The peak 2 enzyme is approximately 100-fold more active than peak 1 toward all substrates tested so far, with a k_{cat}/K_M value of $2.9 \times 10^6\ M^{-1}\ s^{-1}$ for formyl-Met-Leu-*p*-nitroanilide. Third, the two enzymes have different stability. While the peak 1 protein is highly stable under all of the conditions tested, the protein in peak 2 rapidly loses activity at 23 °C ($t_{1/2} \approx 1$ min). Denaturation of the two proteins with guanidinium hydrochloride also gave different curves, with the

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(13) Peptide deformylase from a Phenyl-Sepharose column was quickly passed through a Superdex 75 column equilibrated in a metal-free buffer,[6] and the metal content was determined by inductively coupled plasma (ICP) emission analysis at the University of Georgia.

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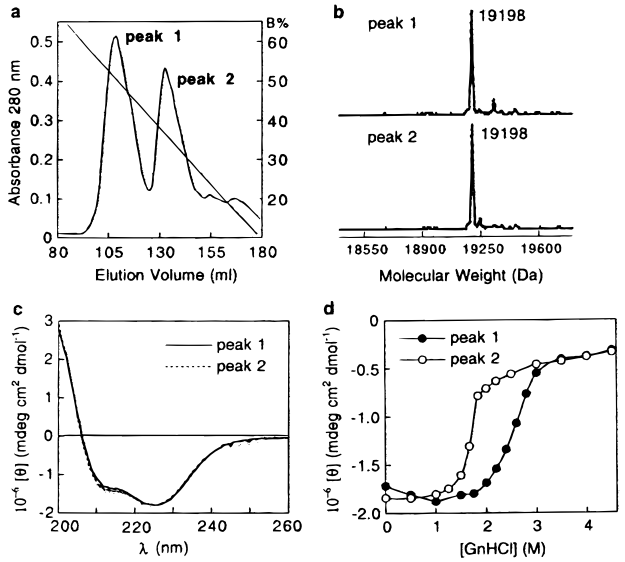

Figure 1. Comparison of the two deformylase forms. (a) Elution profile of a Phenyl-Sepharose column (Pharmacia HR 16/10). The gradient was from 1.7 to 0 M $(NH_4)_2SO_4$ in 20 mM potassium phosphate (pH 7.0) and 10 mM NaCl over 200 mL (4 mL/min). (b) Deconvoluted spectra from LC−ESI MS analysis. (c) Circular dichroism spectra (10 scans) of peak 1 and peak 2 enzymes (1.6 μM) at 20 °C. (d) Denaturation profiles. Deformylase (25 μM final) was added to solutions (V_T = 500 μL) containing 0−4.5 M guanidinium hydrochloride and incubated at 23 °C for 12 h prior to CD measurement at 225 nm.

peak 1 protein unfolded at ≥3 M and the peak 2 protein unfolded at ≥1.7 M denaturant (Figure 1d). The simplest explanation for these observations is that peak 1 is a zinc-containing enzyme as reported by Meinnel et al.,[7] whereas peak 2 contains a different metal ion, most likely iron.

To demonstrate the functional role of iron in peptide deformylase, nine fractions from five independent protein preparations, all of which contained homogeneous deformylase polypeptide as judged by SDS−PAGE and coomassie stain, were analyzed for metal content and catalytic activity. A linear correlation between catalytic activity and iron content was found (Figure 2). No such correlation could be found for any of the other 19 metals tested. These results firmly establish that iron, instead of zinc, is the functional metal in the peak 2 enzyme. Since the peak 2 enzyme resembles in many properties (high activity and lability) the deformylase activity in nonoverproducing *E. coli* strains,[5] the iron-containing deformylase in peak 2 is most likely the physiologically relevant species. The zinc-containing peak 1 protein, with much lower activity, is likely formed during iron depletion caused by overexpression.[16] Interestingly, there is an inverse correlation between activity and zinc content, suggesting that zinc and iron compete for the same metal-binding site in the protein. Thus, the iron is likely coordinated by residues His-132, His-136, and Cys-90 in the protein, although other residue(s) may also serve as metal ligand-(s).

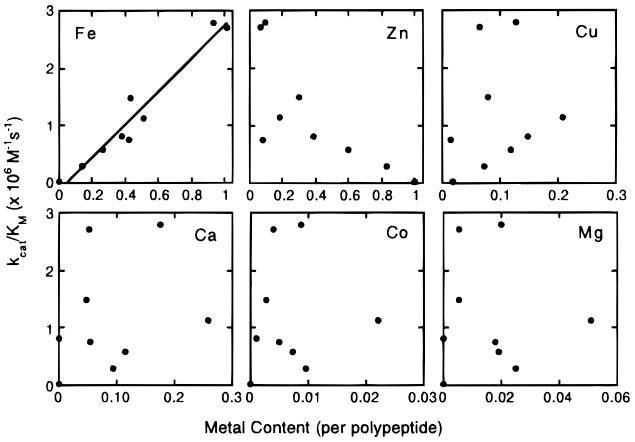

Figure 2. Catalytic activity vs metal content for six divalent metals found in nine protein samples. Other elements tested were Al, B, Ba, Cd, Cr, K, Mn, Mo, Na, Ni, P, Pb, Si, and Sr.

To determine the valence state of the iron metal, freshly purified peak 2 deformylase was acid denatured and the released iron was quantitated by complex formation with 1,10-phenanthroline and ammonium thiocyanate.[17] These experiments showed that the native deformylase contained 0.77 ± 0.02 Fe^{2+} and 0.20 ± 0.03 Fe^{3+} per polypeptide.[18] When the native deformylase was treated with hydrogen peroxide prior to acid denaturation, 0.95 ± 0.03 Fe^{3+} per polypeptide but no Fe^{2+} was found. The peak 2 protein was also examined by electron paramagnetic resonance (EPR) and was found to be EPR silent, consistent with an Fe^{2+} center. Our preliminary results show that peptide deformylase from *Haemophilus influenzae* also contains an iron metal and is highly unstable. Thus, the *E. coli* peptide deformylase, and perhaps deformylases from other organisms as well, is an Fe^{2+} metalloenzyme. The dramatically different catalytic activities associated with the Fe^{2+} vs Zn^{2+} enzymes, which apparently have a similar tertiary structure, provide further support that the metal ions are directly involved in catalysis.[6] To our knowledge, this represents the first example of an iron-containing amidase. Since most of the metalloproteases and metallopeptidases utilize Zn^{2+} as the catalytic metal,[9] the unique structural feature of peptide deformylase will facilitate the design of selective deformylase inhibitors.

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(18) The small amount of Fe^{3+} is likely due to air oxidation of Fe^{2+} during sample handling.