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Enzymatic Properties of *Escherichia coli* Peptide Deformylase

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Since its discovery in crude extracts in the late sixties, *Escherichia coli* peptide deformylase activity could not be further characterized because of an apparent extreme instability. We show that this behavior was caused by an inadequate activity assay, involving substrate concentration inhibition and substrate precipitation in crude extracts. The homogeneous protein, as it was previously purified (T. Meinnel and S. Blanquet J. Bacteriol. 175:7737–7740, 1993), had actually retained its initial activity. The influence on the deformylation reaction of several factors was studied and used to improve the activity assay. Pure peptide deformylase proves to act only on peptide substrates with an *N*-formylmethionyl moiety. In agreement with the occurrence of zinc in the enzyme, peptide deformylase activity is inhibited by 1,10-phenanthroline.

In prokaryotes, the translation apparatus appears to be simpler than that in eukaryotes. For instance, only three specific initiation factors occur in *Escherichia coli*, whereas more than 13 have already been described in mammals. However, prokaryotic translation initiation involves an additional step of formylation of methionyl-$tRNA_f^{Met}$. The occurrence of this modification of the first incorporated methionine, together with the requirement, conserved throughout evolution, for the recycling of the N-terminal methionine (reviewed in reference 15), may explain the need for bacteria to possess a peptide deformylase activity (1, 9, 16). Although earlier characterized in crude extracts, peptide deformylase (EC 3.5.1.27) could not be further studied because of the strong lability of its activity upon any attempt of protein fractionation (1, 9). However, some features of the catalytic mechanism were described. For instance, it was shown that both a deformylated peptide and formate were the products of the hydrolysis (1). Also, a strong specificity towards the *N*-formylmethionyl (Fo-Met) moiety was proposed (1). Notably, Fo-Met-$tRNA_f^{Met}$ was not hydrolyzed, while Fo-puromycin, a compound resembling Fo-Met-Tyr, was a substrate (9). Finally, a strong sensitivity of the deformylase activity to the addition, in crude extracts, of either EDTA or reducing agents, such as 2-mercaptoethanol or dithiothreitol, was noticed. The loss of deformylase activity upon ammonium sulfate precipitation and an "extraordinary instability" at 37°C were also reported (1, 9). Altogether, these results led those previous authors to the conclusion that the catalyzed reaction required an additional cofactor.

Very recently, the gene (*fms* or *def*) encoding *E. coli* peptide deformylase has been characterized (7, 11, 12). As could be expected from the function that deformylase has upstream from the strictly required step of removal of the first methionine (6), this gene proved to be essential for bacterial growth (11, 13). It is noteworthy that *fms* and *fmt*, the genes encoding peptide deformylase and methionyl-$tRNA_f^{Met}$ formyltransferase, respectively, are associated in the same transcriptional unit (7, 12, 14). This genetic linkage is conserved in *Thermus thermophilus* (13). The availability of the *fms* gene allowed us to overproduce the corresponding protein (12). However, using the enzymatic assay described by Adams (1), we met the same difficulties as previous workers and could not retain the deformylase activity upon purification. Some additional biochemical features of the protein could nevertheless be described. The enzyme behaves as a monomer and is strongly associated with one zinc atom. Indeed, a motif characteristic of zinc aminopeptidases of the thermolysin family (HEXXH) could be seen in the amino acid sequences of the peptide deformylase from *E. coli* and from *T. thermophilus* (13).

The present study deals with the enzymatic characterization of fully active homogeneous peptide deformylase.

Peptide deformylase activity is inhibited at a high peptide substrate concentration. In order to search for optimal conditions of the deformylase activity measurements in crude extracts, the Fo-Met-Ala substrate concentration was varied from 1 to 20 mM. Surprisingly, while the deformylation rate linearly increased with the substrate concentration in the range of 1 to 4 mM, it was fourfold lower at 20 mM than at 4 mM. This behavior suggested substrate concentration inhibition. In another set of experiments, the specific rate of deformylation in the presence of 20 mM Fo-Met-Ala was measured at various crude extract concentrations. Unexpectedly, the rate rose from an unmeasurably low value at the lowest crude extract concentration to a value on the order of 0.7 nmol of product $\cdot s^{-1} \cdot mg^{-1}$ of protein at the highest concentration. In parallel with this stimulation of the initial rate, we observed that the substrate (20 mM) precipitated in the assay. From this, we supposed that such a precipitation induced by an increase of the crude extract concentration relieved the enzyme of substrate concentration inhibition thanks to a reduction of the available free substrate. In agreement with results of earlier studies (1, 12), the activity, as measured in the presence of 20 mM substrate, was lost if the crude extract was dialyzed or submitted to ammonium sulfate precipitation prior to the activity measurement. Actually, with the latter crude extracts, precipitation of the substrate (20 mM) no longer occurred. This suggested that precipitation of the substrate was caused by small molecules or ions occurring in the crude extract and which were lost upon the above-described treatments.

Purification of active peptide deformylase to homogeneity. Taking into account the above-described results, the deformylase activity was reexamined at each step of the purification as described previously (12) with a reduced substrate concentration in the assay, i.e., a concentration (i) avoiding precipitation of the peptide even in the presence of the crude extract and (ii)

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TABLE 1. Purification of active peptide deformylase to homogeneity

Purification step	Amt of protein (mg)[a]	Total activity (U)[b]	Sp act (U/mg of protein)	Yield (%)	Relative purification
Crude extract	383.1	880	2.30	100.0	1.00
Streptomycin	311.5	858	2.75	97.5	1.20
Superose	97.5	598	6.13	67.9	2.67
Q-Hiload	41.9	512	12.22	58.2	5.32
Hi-propyl[c]	17.5	233	13.31	26.6	5.81

[a] Assayed as described elsewhere (4) by using the Bio-Rad protein assay kit and bovine serum albumin as the standard.

[b] One unit is defined as the amount of enzyme capable of hydrolyzing 1 nmol of Fo-Met-Ala-Ser per s in the standard assay, i.e., at 37°C with 50 mM HEPES (pH 7.0) and 0.5 M KCl.

[c] This step proved necessary to remove a minor contaminant. Moreover, two supplementary minor peaks with pure peptide deformylase activity were discarded (see text). This explains the low yield of this step.

for which substrate inhibition is not likely to occur. A 4 mM substrate concentration was chosen. Under these new assay conditions, we could verify that peptide deformylase activity was almost fully retained from the beginning to the end of the purification procedure (Table 1). In addition, the enzyme which we previously purified and which we believed to be inactive (12) exhibited full deformylation activity under such assay conditions. Several independent purifications of peptide deformylase were carried out from JM101Tr cells carrying the pUCdef plasmid as previously described (12), except that a 3% (wt/vol) streptomycin sulfate concentration was used in the preparation of the crude extract. During the last chromatographic step of the purification of peptide deformylase on a hydrophobic interaction column, we noticed, however, that two minor peaks of activity (II and III) appeared in an irreproducible manner along the elution profile after a first major peak (I) which always contained at least 70% of the applied protein. This behavior was independent of the addition of 2-mercaptoethanol. When subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, the proteins corresponding to the three peaks comigrated. Moreover, they displayed similar peptide deformylase specific activities. By using antibodies raised against form I (unpublished results), a very similar reactivity of the three forms was observed, according to Western blot (immunoblot) analysis. In addition, matrix-assisted laser desorption mass spectrometry indicated that the three forms had the same molecular mass of 19,200 ± 30 Da. All these experiments strongly suggested that form I, form II, and form III were very close species and derived from the same polypeptide chain. However, because of the irreproducible character of the occurrence and amounts of forms II and III, we did not study them any further and discarded them at the end of the purifications. The characterization of form I, already identified by N-terminal sequencing and mass spectrometry (12), is shown in Table 1.

Optimization of the peptide deformylase assay. The availability of a homogeneous active peptide deformylase sample allowed us to optimize the activity assay as described by Adams (1). Because of the relatively low sensitivity of the ninhydrin coloration, initial rates of deformylation could not be determined at substrate concentrations lower than 1 mM. In addition, as expected from the results of the first section, deformylation could not be detected at peptide concentrations above 20 mM. Significant deformylation rates occurred, however, at lower concentrations, with a maximal rate (0.1 s^{-1}) corresponding to about 6 mM substrate (Fig. 1). Moreover, the

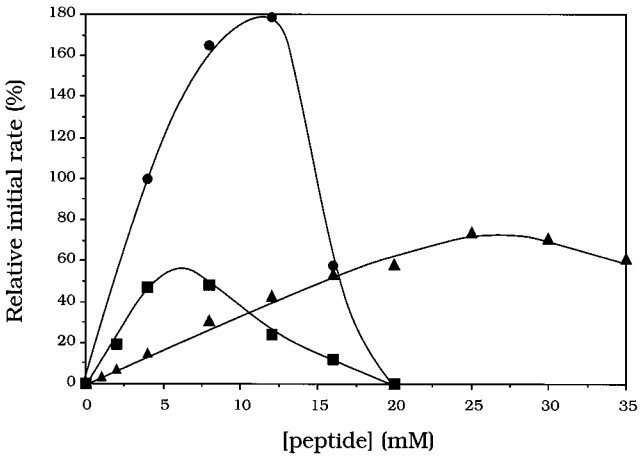

FIG. 1. The initial rate of deformylation depends on the concentration and the nature of Fo-Met peptide substrates. The initial rates of deformylation were measured as a function of increasing peptide concentrations. Squares, Fo-Met-Ala; circles, Fo-Met-Ala-Ser; triangles, Fo-Met-Ser-Asn-Glu. A relative rate of 100 was assigned to the value obtained in the presence of 4 mM Fo-Met-Ala-Ser (0.33 s^{-1}).

deformylation rate linearly increased from 1 to 4 mM peptide, meaning thereby that the K_m value was higher. This K_m as well as the maximal velocity could not, however, be measured because of the substrate concentration inhibition effect (Fig. 1). The deformylation of other peptides was therefore examined (Table 2 and Fig. 1). In agreement with the proposed selectivity of the enzyme with respect to the Fo-Met moiety, neither *N*-acetyl-Met-Ala-Ser nor Fo-Ala-Gly-Ser-Glu could be hydrolyzed. Fo-Met was not a substrate either, meaning thereby that at least two amino acids were required for the deformylation to occur at a measurable rate. However, the nature of the side chain of the second amino acid appeared not to be very important since peptides with lysine, alanine, or valine were hydrolyzed with very close deformylation rates (Table 2). Finally, the rate of deformylation was sensitive to the length or to the hydrophilic character of the peptide since the tripeptide Fo-Met-Ala-Ser was a more efficient substrate than the tetrapeptide Fo-Met-Ser-Asn-Glu (Table 2 and Fig. 1). As shown in Fig. 1, the rate of deformylation of the tripeptide Fo-Met-Ala-

TABLE 2. Effect of the composition of a peptide substrate on its initial rate of hydrolysis by peptide deformylase

Peptide[a]	Relative rate[b]
Fo-Met	<0.1
Fo-Met-Ala	47 ± 5
Fo-Met-Lys	45 ± 5[c]
Fo-Met-Val	38 ± 4
Fo-Met-Ala-Ser	100 ± 10
Fo-Met-Ser-Asn-Glu	15 ± 2
Ac-Met-Ala-Ser	<0.1
Fo-Ala-Gly-Ser-Glu	<0.1

[a] Peptides were purchased from Bachem AG, Serva, or Sigma. The initial peptide concentration in the assay was 4 mM. Ac, *N*-acetyl.

[b] Initial rates of deformylation were measured in the standard buffer, i.e., at 37°C with 50 mM HEPES (pH 7.0) and 0.5 M KCl. A value of 100 was given to the initial rate of deformylation obtained with peptide Fo-Met-Ala-Ser (i.e., 0.33 s^{-1}). A value of <0.1 means that no hydrolysis with a relative rate greater than 0.1 could be detected.

[c] A stronger offset of the colorimetric assay was caused by the additional reactivity of the free amino group of lysine. The value for this peptide was not, however, affected and can be compared with the other values.

Ser, with a maximal initial rate of 0.5 s^{-1} reached at 8 mM, was significantly higher than that of the corresponding shorter peptide (Fo-Met-Ala). Inhibition at a high peptide concentration (beyond 12 mM), however, still occurred, and deformylation could no longer be detected above 20 mM. Notice that the concentration at which peptide concentration inhibition appeared increased with the length of the peptide (Fig. 1). Fo-Met-Ala-Ser was selected as the substrate of our further studies.

The effects of pH, temperature, and ionic strength were assessed in a second step (Fig. 2). In the analysis, buffers with a primary amino group such as Tris were avoided because of the interference with ninhydrin in the assay. Deformylase activity remained roughly constant within a large pH range, i.e., from pH 6.5 to at least pH 11.2. Concerning temperature, a constant increase of the initial rate of deformylation was observed from 25 to 60°C. Beyond 60°C, the activity dropped, probably reflecting the thermal inactivation of the enzyme. A twofold increase of the initial rate was observed when the KCl concentration was raised from 0 to 0.5 M. Beyond 0.5 M KCl, the deformylation rate still increased, but to a smaller extent. Because peptide deformylase is a zinc protein (12), we searched for a possible effect of the addition of this metal in the activity assay. The deformylase activity was not improved by small ZnCl concentrations. However, at high metal concentrations, inhibition occurred with a K_I of 35 mM. Finally, the activity of peptide deformylase was not sensitive to previous incubation of the enzyme with 20 mM dithiothreitol or 10 mM 2-mercaptoethanol at 4°C for 9 h.

Using this information, we chose the following standard conditions for the assay of peptide deformylase activity: 37°C; 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid sodium salt)-HCl, pH 7.0; [KCl] = 0.5 M; and [Fo-Met-Ala-Ser] = 4 mM. Notice that this concentration of the peptide substrate in the assay is well below the K_m, which should be greater than 10 mM.

Zinc is located within or close to the active center of peptide deformylase. The effect on deformylase activity and zinc binding of several agents known to strongly complex divalent cations (2) was studied. Before measurement of the activity at a given concentration of an agent, the enzyme was preincubated for 6 h in the presence of the same concentration of this agent. Neither EDTA (up to 250 mM) nor dipicolinic acid (up to 100 mM) inhibited the deformylase activity. The insensitivity of pure enzyme to EDTA as well as to thiols (see above) contrasts with previous reports (1, 12) stating that these compounds decreased the deformylation rate in crude extracts. The latter experiments with crude extracts were assayed at a high peptide concentration. We hypothesize therefore that these compounds might have indirectly modified the rates measured under these conditions.

Moreover, the stoichiometry between zinc and the enzyme was unchanged after a 1-week dialysis against a buffer containing 10 mM EDTA or 20% (wt/vol) Chelex 100 (Bio-Rad). This latter resin is routinely used in the preparation of apoenzymes devoid of zinc (18). Contrarily, 8-hydroxyquinoline (Fluka) at 2 mM, a concentration close to saturation, inhibited 50% of the deformylation activity. With 1,10-phenanthroline (Sigma), complete inhibition could be achieved at 5 mM with an inhibition constant of 0.63 mM (Fig. 3). After extensive dialysis of peptide deformylase for 1 week against a buffer containing 6 mM 1,10-phenanthroline, zinc remained, however, bound to the enzyme. Finally, the nonchelating structural analog of 1,10-phenanthroline, 4,7-phenanthroline (Aldrich), added up to 5 mM, had no effect on the activity of peptide deformylase (Fig. 3). These experiments indicated that a divalent metal cation

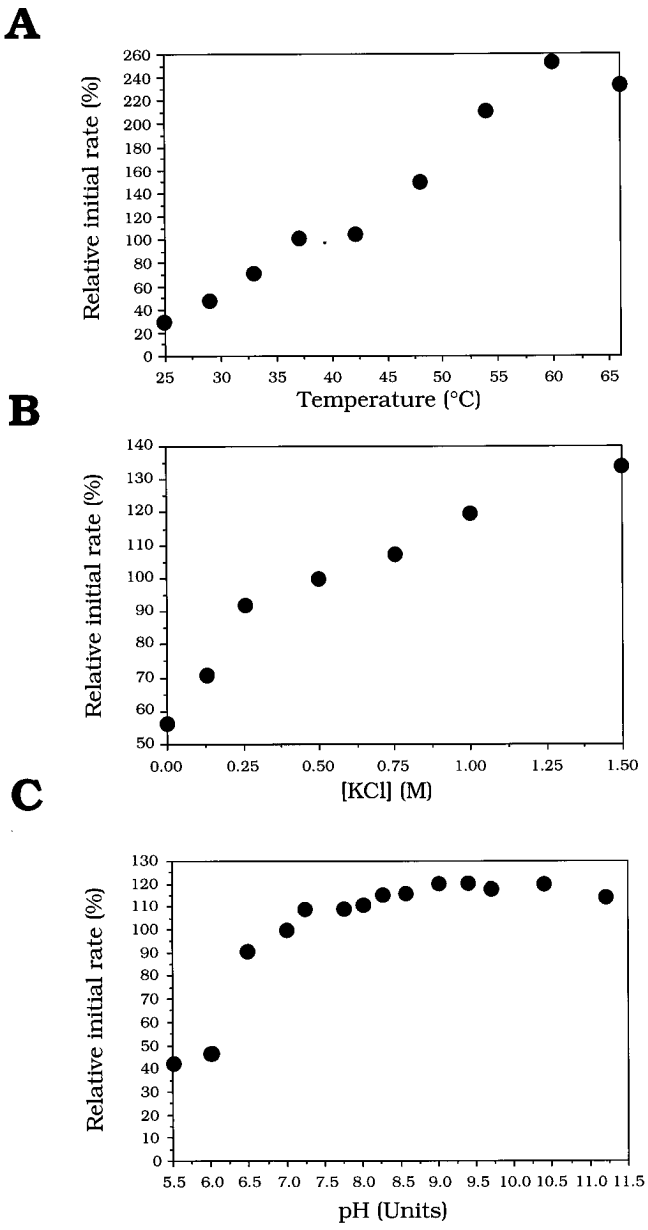

FIG. 2. Influences of temperature, ionic strength, and pH on the initial rate of hydrolysis by peptide deformylase. A relative rate of 100 was assigned to the initial rate value obtained in the standard assay i.e., at 37°C, in the presence of 50 mM HEPES (pH 7.0) and 0.5 M KCl. The initial rates of deformylation were measured at various temperatures (A) or at various KCl concentrations (B). (C) The initial rate of deformylation was measured in the standard conditions except that the nature of the buffer (50 mM) was as follows: sodium acetate (pH 5.5), HEPES-HCl (Sigma) from pH 6.0 to pH 8.3, *N*,*N*-bis(2-hydroxyethyl)glycine (bicine; Fluka)–NaOH from pH 8.5 to pH 9.4, or 3-(cyclohexylamino)propane sulfonic acid (Serva)–NaOH from pH 9.7 to pH 11.1.

strongly associated to the enzyme could be complexed by 1,10-phenanthroline. To know whether metal ions other than zinc could be involved, the occurrence of 23 different metals (Ag, Al, As, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Mo, Ni, P, Pb, Si, Sn, Ti, Zn, and Zi) was assessed by using inductively coupled torch plasma emission spectrometry (20). For this purpose, a 60 μM sample of the enzyme was first extensively dialyzed against 10 mM Tris-HCl (pH 7.5). While the zinc concentration was found stoichiometric with that of the pro-

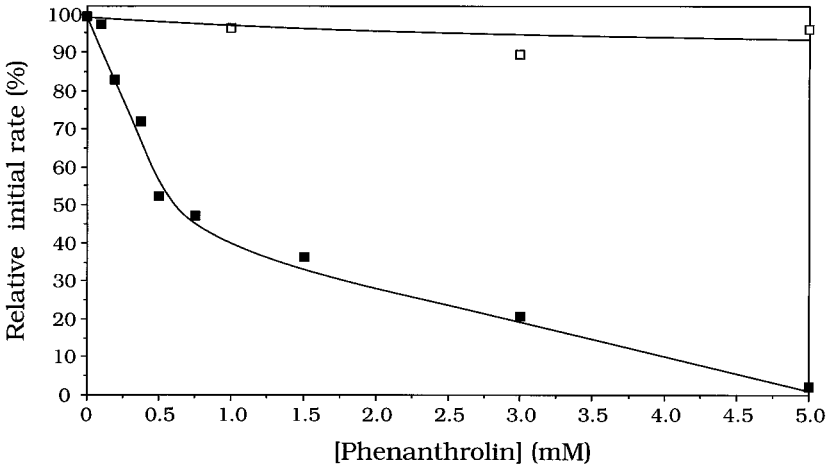

FIG. 3. Inhibition of peptide deformylase activity by 1,10-phenanthroline. Prior to the assay, pure enzyme was incubated for 6 h on ice in the presence of the indicated phenanthroline concentration. Enzymatic activity was then assayed in the standard buffer containing the same concentration of 1,10-phenanthroline (black squares) or 4,7-phenanthroline (open squares).

tein, the concentrations of each of the other metals were less than 1 μM in both the control dialysis buffer and the protein sample. This result strongly suggested that zinc was the only metal ion occurring in peptide deformylase and that, consequently, it was the target of 1,10-phenanthroline in or near the active center of the enzyme.

Concluding remarks. We show that the previously reported apparent lability of the enzyme during purification was caused by an inadequate activity assay (1) and that the homogeneous enzyme that we had already purified (12) was actually 100% active. The availability of an improved enzymatic assay with pure and active peptide deformylase allowed us to reexamine its selectivity towards various substrates. As already suggested by Adams (1), the enzyme needs both a Fo and a methionine moiety in the peptide substrate. In addition, it requires a second amino acid beyond the first methionine. This conclusion is also supported by the previous demonstration that Fo-Met-tRNA$_f^{Met}$ is protected from deformylation (9). Peptide deformylase appears to work therefore as a peptidase, removing the N-terminal formyl group.

That peptide deformylase resembles a peptidase is also supported by the occurrence of an HEXXH motif characteristic of the family of zinc metallopeptidases (12). The most documented member in this family is thermolysin (3, 17). Within the family, the side chains of the two histidines of the motif participate in the binding of the metal ion. The conserved glutamate does not bind zinc but is essential for the catalysis. Moreover, zinc may be easily removed with the help of several chelating agents, including 1,10-phenanthroline, and the apoenzyme was shown to be inactive (8). Zinc, which is therefore located in the active center and directly participates in the enzymatic reaction, plays a catalytic role. That peptide deformylase contains zinc and resembles a protease is not, however, enough to establish that it is a member of the above-described family. In this context, a major distinctive feature of peptide deformylase is the effect of 1,10-phenanthroline, which fails to remove the zinc, although it inhibits the deformylation reaction. Interestingly, a similar behavior of phenanthroline has already been reported in the case of several enzymes (5, 10, 18, 19). In each studied case, the formation of a ternary complex involving zinc, phenanthroline, and the catalytic center of the enzyme was evidenced. By analogy, this may indicate that zinc is part of the catalytic center of peptide deformylase as in the enzymes of the thermolysin family. However, the zinc ion would be much more strongly associated to peptide deformylase than to proteases of the above-mentioned family.

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