A Mutated Bovine Prochymosin Zymogen Can Be Activated without Proteolytic Processing at Low pH*

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As a first step towards understanding how the zymogen structure of prochymosin contributes to the process by which active enzyme is produced, we altered the nucleotide sequence which encodes the amino-terminal (or propeptide) region of the protein. Of the two sites for autoproteolysis of prochymosin, one where prochymosin is formed at a pH of 2 and the other where chymosin is formed at pH 4–5, we changed the former by removing one codon and changing two other codons. This genetically modified prochymosin was proteolytically processed and activated normally at pH 4.5. However, at pH 2 we observed only partial activation of the zymogen and found no evidence of proteolytic processing. The properties of this engineered prochymosin suggest that zymogen activation does not require proteolysis and that the two different zymogen processing sites can function independently from one another.

Bovine prochymosin is the zymogen form of chymosin (EC 3.4.23.4), an aspartyl protease which can coagulate milk. Normally, the zymogen is autocalytically and irreversibly activated by the proteolytic removal of a portion of its NH2-terminal sequence in either of two ways: at pH 4.5 the activation reaction produces chymosin by removal of a propeptide (or activation peptide) and comprising prochymosin residues 1–43; or at pH 2 the activation reaction produces a pseudochymosin product by removal of a propeptide comprising prochymosin residues 1–27. Once formed, pseudochymosin is relatively stable at a pH below 3 or above 6 but is further processed to chymosin when incubated at pH 4.5 (1, 2). Both of these proteins are efficient milk-clotting enzymes.

To help understand how zymogen structure contributes to this activation process, we have begun protein engineering experiments to study the effects of amino acid changes on proteolytic processing and enzymatic activation. This task has been simplified because the prochymosin gene has been cloned, sequenced, and expressed in Escherichia coli (3–5). In particular, we constructed a plasmid which encodes a prochymosin having several amino acid changes in the region of Phe2′-Leu28, the site where prochymosin is processed at pH 2 involved in the activation of prochymosin to pseudochymosin. We sought to change the codons immediately following Phe2′ by insertion of a synthetic oligonucleotide at the PstI site.

Fig. 1 summarizes in more detail the construction of the plasmid which encodes the altered prochymosin. Plasmid pHWA43 was linearized by digestion with PstI, treated with mung bean nuclease to form blunt-ended DNA, and then ligated to a double-stranded synthetic oligonucleotide, 5′-phosphorylated dCTCTAGAG, containing an XbaI site. After ligation this mixture was used to transform E. coli strain MH1 to ampicillin resistance and gave rise to a new plasmid, pDB10, that contained the new XbaI site. Our objective was to replace 4 base pairs of the original PstI site with an 8-base pair linker carrying a unique restriction site for identification. From this intermediate we would remove 4 base pairs from the new XbaI site to restore the proper reading frame. Nucleotide sequencing of the pDB10 plasmid showed that three additional nucleotides had been unexpectedly lost at the 3′ end of the PstI site during its construction. (The loss of additional nucleotides is most likely due to double strand nuclease activity which we have found in some commercial lots of mung bean nuclease.) Thus, the intermediate plasmid, pDB10, had lost one codon which could not be restored and would cause the final plasmid construction to be one codon shorter than the parental pHWA43 plasmid. Finally, to restore the original reading frame, plasmid pDB10 was linearized with XbaI, digested with mung bean nuclease, and reci-

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MATERIALS AND METHODS

Bacterial Strains and Media—The E. coli strains used were JM83, MM294, M1H (from J. Hedgpeth, this institution), and CY15001 (from C. Yanofsky, Stanford University). Cells were grown on Luria broth (liquid and solid media) and, when necessary, supplemented with ampicillin (Sigma) at 150 μg/ml.

Enzymes and Oligonucleotides—Restriction endonucleases PstI and XbaI were obtained from New England Biolabs. Mung bean nuclease, T4 DNA ligase, and polynucleotide kinase were purchased from Pharmacia P-L Biochemicals. The synthetic oligonucleotide (dCTCTAGAG, containing an XbaI site) was obtained from Biosearch.

Plasmid DNA Manipulation—The isolation of bacterial plasmid DNA and the preparation and transformation of E. coli cells with ligated DNA was performed by standard techniques (6). DNA sequencing was performed according to the method of Maxam and Gilbert (7).

Renaturation of Prochymosin Synthesized in E. coli—E. coli cells containing prochymosin were lysed by sonication and then extracted with Triton X-100 (8% final concentration) for 18 h at 4 °C. After centrifuging at 13,000 × g for 15 min, a white pellet was obtained (containing the prochymosin (5)). The detergent-insoluble pellet containing the prochymosin was diluted to a protein concentration of 180 μg/ml in 5 m urea, solubilized by stirring for several hours, and then renatured by diluting the area-solubilized material into 100 volumes of pH 11 phosphate buffer for 10 min followed by acidification (over a 5-min period) to pH 8.5.

RESULTS

Genetic Modification of Bovine Prochymosin—The starting point of generating genetic variants of prochymosin is plasmid pHWA43 which encodes methionyl-prochymosin and directs the synthesis of the zymogen in E. coli (5). This plasmid contains a unique PstI site partially contained within the codons specifying Phe2′-Leu28, the site of proteolytic cleavage at pH 2 involved in the activation of prochymosin to pseudochymosin. We sought to change the codons immediately following Phe2′ by insertion of a synthetic oligonucleotide at the PstI site.

Fig. 1 summarizes in more detail the construction of the plasmid which encodes the altered prochymosin. Plasmid pHWA43 was linearized by digesting with PstI, treated with mung bean nuclease to form blunt-ended DNA, and then ligated to a double-stranded synthetic oligonucleotide, 5′-phosphorylated dCTCTAGAG, containing an XbaI site. After ligation this mixture was used to transform E. coli strain MH1 to ampicillin resistance and gave rise to a new plasmid, pDB10, that contained the new XbaI site. Our objective was to replace 4 base pairs of the original PstI site with an 8-base pair linker carrying a unique restriction site for identification. From this intermediate we would remove 4 base pairs from the new XbaI site to restore the proper reading frame. Nucleotide sequencing of the pDB10 plasmid showed that three additional nucleotides had been unexpectedly lost at the 3′ end of the PstI site during its construction. (The loss of additional nucleotides is most likely due to double strand nuclease activity which we have found in some commercial lots of mung bean nuclease.) Thus, the intermediate plasmid, pDB10, had lost one codon which could not be restored and would cause the final plasmid construction to be one codon shorter than the parental pHWA43 plasmid. Finally, to restore the original reading frame, plasmid pDB10 was linearized with XbaI, digested with mung bean nuclease, and reci-
circularized by blunt-end ligation. Transformed cells containing plasmid DNA, which could not be cut with XbaI, were cultured and their proteins were analyzed by polyacrylamide gel electrophoresis. In this way, we identified plasmid pDB20 which encoded a prochymosin-sized protein (calf prochymosin) not seen in lysates of untransformed cells. Nucleotide sequencing of plasmid pDB20 showed that the wild-type prochymosin sequence had been changed from \(-\text{Phe}^{27}\)-\text{Leu}^{28}\)-\text{Gln}^{29}\)-\text{Lys}^{30}\)-Gln^{31} (8) to -\text{Phe}^{27}\)-\text{Pro}^{28}\)-Arg^{29}\)-Gln^{30} (Fig. 1b). Thus, the modified prochymosin differs from pWHA43 prochymosin by the loss of Lys^{30}, the substitution of proline for leucine at position 28, and the substitution of arginine for glutamine at position 29.

Plasmid pDB20 Encodes an Insoluble Prochymosin Protein in E. coli—The altered pDB20 prochymosin has several properties in common with the [Met]prochymosin encoded by pWHA43. In E. coli, plasmid pDB20 directs the synthesis of a protein which comigrates with calf stomach prochymosin and has a molecular weight of 40,500, as determined by sodium dodecyl sulfate-gel electrophoresis. Unlike authentic calf prochymosin, which is a soluble protein, the pDB20 prochymosin is located in the insoluble fraction of the E. coli lysate, as is the pWHA43 prochymosin (5). Similarly, it is not solubilized in solutions containing nonionic detergents or high salt concentrations. The two insoluble proteins produce no milk-clotting activity when assayed as crude lysates of E. coli even after preincubation at acid pH, indicating that, like the pWHA43 prochymosin, the pDB20 prochymosin is also synthesized in an inactive form.

In order to further characterize the genetically modified zymogen, the insoluble pDB20 prochymosin was solubilized in 8 M urea (as described under “Materials and Methods” and in Refs. 5 and 8) and then renatured to a form that was soluble and could be activated by acid pH, by diluting the urea-solubilized prochymosin into a phosphate buffer at pH 11 for 10 min and then by a gradual acidification to pH 5.5. We found that the conditions required for optimal solubilization and renaturation of the pDB20 and pWHA43 prochymosins are identical.

Acid-catalyzed Activation of the pDB20 Prochymosin—The solublezymogen form of the pDB20 prochymosin, like authentic calf prochymosin, had no significant milk-clotting activity prior to its activation at acid pH (<0.05% that of fully activated material). When incubated at pH 4.5, the pDB20 prochymosin exhibited a slow rate of increase in milk-clotting activity (sigmoidal activation kinetics), which closely resembled the rate of activation of the parental pWHA43 prochymosin (Fig. 2a). Normally, the appearance of milk-clotting activity at pH 4.5 results from the autocatalytic conversion of prochymosin to chymosin which removes residues 1–43 of the zymogen. Fig. 2b shows that pDB20 prochymosin, activated at pH 4.5, was indeed processed to a product with the same electrophoretic mobility as calf chymosin. This activation product was concentrated and purified by preparative gel electrophoresis and its amino acid sequence was determined by the method of Hunkapiller et al. (9). The results (see Table I) indicated an amino-terminal sequence of Gly-Glu-Ala-Ser-Val—the same as for authentic chymosin as reported by Foltmann et al. (10). Activity measurements of both pDB20 and pWHA43 prochymosins activated at pH 4.5 indicated that the milk-clotting specific activities of the two proteins were virtually the same (less than 10% difference), both with respect to activity/mg of bacterial cell paste as well as activity/mg of renatured protein. Previously, we have demonstrated that purified pWHA43 and calf chymosins also have the same milk clotting specific activities (5).

In contrast, the generation of milk-clotting activity by activation of pDB20 prochymosin at pH 2 occurred in a manner quite different from that of the parental prochymosin activated at this pH. Normally, calf prochymosin shows hyperbolic activation kinetics at pH 2 and activates at a much faster rate than at pH 4.5 (1). However, the kinetics of activation of pDB20 prochymosin during the first several hours of incubation at pH 2 were not rapid and hyperbolic but were slow, sigmoidal, and quite similar to the pH 4.5 activation kinetics of this zymogen. In contrast, the parental pWHA43 prochymosin exhibits the typical hyperbolic activation curve at pH 2. The maximum activity produced by the

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**Fig. 1. Construction of prochymosin expression plasmid pDB20.** a, major steps in the construction of plasmid pDB20. b, Comparison of the NH\(_2\)-terminal amino acid sequence of the parental pWHA43 prochymosin (5) and that predicted for the pDB20 prochymosin from its DNA sequence. Solid arrow indicates cleavage site to produce pseudochymosin; open arrow indicates cleavage site to produce chymosin.
produces pseudochymosin (left panel) which has milk-clotting activity equal to chymosin (5). The pDB2O prochymosin was incubated with electrophoresis on a 12.5% acrylamide-sodium dodecyl sulfate slab staining (right panel). Samples were 4 pg of calf stomach prochymosin pH 4.5 activated pDB2O prochymosin was analyzed with repetitive cycles (left panel) or 4.5 (right panel). The incubation of prochymosin at pH 2 for up to 96 h did not change the level of milk-clotting activity nor was there evidence for zymogen processing. The results in Fig. 2b indicate a significant reduction in silver-staining protein following acid activation at both pH 2 and 4.5. In our experience, this is typical for renatured recombinant prochymosins and reflects the general proteolytic action of active chymosin on improperly renatured prochymosin molecules. The remaining protein appears to be enzymatically active and stable for many weeks after preparation.

In a similar fashion, we have found that at pH 6.5 trypsin can fully process and thus activate the wild-type and variant prochymosins as well as degrade improperly renatured molecules (see Fig. 2). The pDB20 prochymosin activated at pH 2 shows no additional protein loss upon incubation at pH 4.5 or trypsin treatment at pH 6.5.

The amino-terminal sequence was determined for the 48-h, pH 2 activation product, after its purification by preparative gel electrophoresis, and is shown in Table I. This sequence (Ala-Glu-Ile-Thr-Agr-Ile-Pro-Leu) is unique and unambiguous within the prochymosin structure. The pWHA43 prochymosin also has this same amino-terminal sequence prior to its activation and thus indicates that the initiator methionine is absent from both insoluble prochymosins isolated from E. coli (5).

These results suggest that the pDB20 prochymosin can be partially activated without proteolytic processing of the zymogen. Since the stained acrylamide gels indicate a similar recovery of protein after pH 2 or 4.5 activation, the loss of protein after activation is independent of processing. The measured milk-clotting activity is consistent with the amount of protein visible after activation. These results would appear to preclude the possibility that the unprocessed protein observed on gels and by sequencing is attributable to inactive prochymosin or that the pH 2 activated component of the pDB20 prochymosin could be attributed to a small amount of cleaved prochymosin.

**Fig. 2. Activation and processing of DB20 prochymosin at acid pH.** a, Solutions of renatured pWHA43 and pDB20 prochymosins were adjusted to identical protein concentrations and then activated by incubation at either pH 2.0 (left panel) or 4.5 (right panel). Chymosin activity was assayed at various times using a milk-clotting assay. The specific activity of pure chymosin corresponds to 2900 units/mg of protein. b, Purified protein samples were fractionated by electrophoresis on a 12.5% acrylamide-sodium dodecyl sulfate slab gel (14) and detected by Coomassie Blue (left panel) or by silver staining (right panel). Samples were 4 µg of calf stomach prochymosin after incubation as labeled (left panel) and equal volume loadings (50 µl) of pDB20 prochymosin (at 180 µg/ml) after incubation as labeled (right panel). The incubation of prochymosin at pH 2 normally produces pseudochymosin (left panel) which has milk-clotting activity equal to chymosin (5). The pDB20 prochymosin was incubated with trypsin at a mass ratio of 1/4.

**Table I**

| Cycle No. | pH 2 activated | pH 4.5 activated |
|-----------|----------------|-----------------|
|           | PTH Yield<sup>a</sup> | PTH Yield<sup>a</sup> |
|           | pmol | pmol |
| 1         | Ala  | 295 | Gly |
| 2         | Glu  | 308 | Glu  | 61 |
| 3         | Ile  | 232 | Val  | 43 |
| 4         | Thr<sup>b</sup> | ND<sup>c</sup> | Ala | 44 |
| 5         | Arg<sup>d</sup> | Ser<sup>e</sup> | ND |
| 6         | Ile  | 205 | Val  | 33 |
| 7         | Pro  | 163 |     |    |
| 8         | Leu  | 204 |     |    |

<sup>a</sup> Calculated from peak height of high performance liquid chromatography.

<sup>b</sup> Identified qualitatively with PTH and PTH-dehydro derivatives.

<sup>c</sup> ND, not determined.

<sup>d</sup>,<sup>e</sup> not identified.

DISCUSSION

Site-specific mutagenesis of a structural gene is a powerful tool for helping to elucidate structure-function relationships of proteins. To enhance our understanding of zymogen activation, we have generated and characterized an altered acid protease zymogen of bovine prochymosin. This recombinant protein produced in E. coli differs from parental bovine prochymosin by three contiguous amino acids in the amino-terminal propeptide. This variant resembles the parental prochymosin in several other respects. In addition to having the same electrophoretic mobility on sodium dodecyl sulfate gels, it is also produced in an insoluble and inactive state within E. coli and can be solubilized and readily renatured by the same conditions developed for recombinant [Met]prochymosin produced in bacteria (5, 8). In addition, the variant prochymosin displays kinetics of activation at pH 4.5 identical with the wild-type prochymosin. Thus, the prodromosin can be partially activated without proteolytic processing of the zymogen. Since the stained acrylamide gels indicate a similar recovery of protein after pH 2 or 4.5 activation, the loss of protein after activation is independent of processing. The measured milk-clotting activity is consistent with the amount of protein visible after activation. These results would appear to preclude the possibility that the unprocessed protein observed on gels and by sequencing is attributable to inactive prochymosin or that the pH 2 activated component of the pDB20 prochymosin could be attributed to a small amount of cleaved prochymosin.
to those of the parental material and its processing appears normal. Thus, the described amino acid changes in the pro-
peptide in no way affect activation or proteolytic cleavage of the pH 4.5 processing site which is downstream from the altered pseudochymosin processing site.

However, major differences appear between these proteins during activation at pH 2.0. Not only is the activation of the variant zymogen less extensive and follows different kinetics than the parental molecule, but also the activity that is generated in the variant, which can amount to almost 50% of the parental material, occurs in the absence of proteolytic processing. Pedersen et al. (1) have noted a brief burst of enzyme activity which precedes the initial processing of calf prochymosin activated at pH 2. The lack of proteolytic processing of the altered prochymosin at pH 2 could be due to the introduction of a proline residue next to the phenylalanine at the site of pH 2 catalyzed proteolysis and/or to the concomitant one-amino acid deletion in the propeptide which could shift the position of the pseudochymosin processing site within the zymogen structure. Previous substrate specificity studies of chymosin (11, 12) have focused on analogs of ε-casein peptides but did not investigate amino acid changes at the actual site of substrate proteolysis. Prochymosin autoactivation at either pH 2 or 4.5 as well as chymosin-catalyzed cleavage of ε-casein during milk clotting has been shown to involve proteolytic cleavage between a phenylalanine residue and either a hydrophobic or nonpolar residue. There is not sufficient structural data in the literature to ascertain whether the engineered changes in amino acid sequence have altered the regional secondary structure of the pDB2O zymogen pro-
the engineered changes in amino acid sequence have altered pseudochymosin processing site.

Thus, pDB20 prochymosin activation properties may also change with increasing initial protein concentrations. Because the activation of the modified prochymosin at pH 2 occurs in the absence of proteolytic processing, it may be possible to reverse the activation reaction. We are pursuing this objective.

Results such as these should help clarify the role of specific amino acids in the zymogen activation process. Using genetic tools to substitute or reposition residues of the protein, followed by testing its functional properties, will greatly enhance our understanding of structure-function interactions of prochymosin.

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