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Cytoplasmic Inclusion Bodies in *Escherichia coli* Producing Biosynthetic Human Insulin Proteins

Abstract. Escherichia coli *that has been genetically manipulated by recombinant DNA technology to synthesize human insulin polypeptides (A chain, B chain, or proinsulin) contains prominent cytoplasmic inclusion bodies. The amount of inclusion product within the cells corresponds to the quantity of chimeric protein formed by the bacteria. At peak production, the inclusion bodies may occupy as much as 20 percent of the* Escherichia coli *cellular volume.*

Manipulation of the bacterial genome through recombinant DNA technology to produce organisms capable of forming pharmacologically useful peptides is an area of intense research activity. As part of an ongoing project for the production of human insulin in bacteria, we have examined cultures of *Escherichia coli*, using scanning and transmission electron microscopy at intervals during the culture cycle for the production of insulin chain polypeptides. We have observed a morphologically characteristic intracellular product whose accumulation in the form of inclusion bodies corresponds to the formation of chimeric protein. The product was present in cells bearing plasmids containing gene sequences coding for insulin A chain, insulin B chain, or proinsulin fused to portions of either the lactose or the tryptophan operons. Similar inclusion bodies were not evident in the nonplasmid-containing strain of *E. coli* or in *E. coli* containing the parental plasmid vector pBR322.

The construction of plasmids coding for the expression of insulin A and B chains has been described (*1*). These plasmids contain DNA sequences coding for A or B chain fused to the major portion of the β-galactosidase gene from λ plac 5. In addition, a second series of plasmids in which the β-galactosidase gene fragment has been replaced with an analogous fragment of the tryptophan operon has been constructed. In this series, the formation of product is under the control of the trp promoter, and the chimeric protein consists of insulin chains (A chain, B chain, or proinsulin) fused at the COOH-terminus of a tryptophan operon gene product.

Escherichia coli containing fused gene-bearing plasmids were cultivated in shake flasks in either L broth with ampicillin (500 μg/ml) or in modified M-9 salts medium containing tetracycline (1.5 μg/ml). Growth was obtained with vigorous agitation at 37°C. When the appropriate cell density was achieved, the culture was used to inoculate similar medium in a small fermentor. Samples were taken at intervals throughout the fermentation for evaluation. Typically, cultures grown under these conditions completed the logarithmic phase of growth in about 15 hours. The insulin chimeric proteins were produced as insoluble products by the bacterial cell. They accumulated within the cell, with peak levels being reached at varying times depending on which chimeric protein was being formed and on culture conditions.

Samples taken during fermentation for morphological study were monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis for chimeric protein production. The band identified as chimeric protein increased in density as the fermentation progressed (Fig. 1). The morphology of *E. coli* producing the insulin chain chimeric proteins is illustrated in Figs. 2 to 4. The structure of the chimeric protein producing cells was distinct from that of the nonplasmid-containing cells (Fig. 5) in that a large proportion of the cell was filled with a finely granular, moderately electron-opaque material. The material lacked an obvious boundary such as an enclosing membrane or the "membrane coat" that has been observed with poly-β-hydroxybutyrate and polyglucose granules (*2*). The product, nevertheless, was segregated from the rest of the bacterial cytoplasm. At moderately high magnifications (> 150,000×) fine fibers with diameters in the 2- to 4-nm range could be resolved within some areas of the granular matrix. We have consistently observed inclusion bodies in *E. coli* cultures producing the insulin chain chimeric proteins. On the basis of our observations with transmission electron microscopy and with light microscopy by dark-field and differential

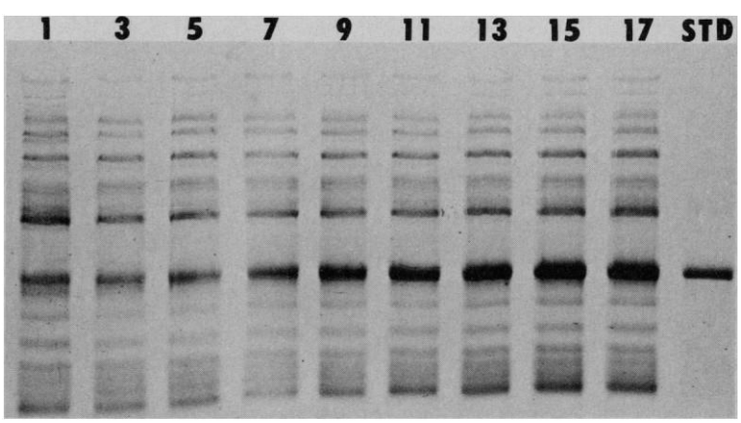

Fig. 1. Sodium dodecyl sulfate (SDS) gel electrophoresis of chimeric protein. Broth samples were collected at the stated times after initiation of fermentation and the cells were centrifuged. The supernatant was removed and the cell pellet was frozen. The pellet was dissolved in a buffered solution of 2.3 percent SDS and 5 percent β-mercaptoethanol and heated at 90° to 100°C for 5 minutes (*8*). A portion of the sample was analyzed with a 7.5 percent polyacrylamide gel prepared wth imidazole buffer (*p*H 7.35) containing 0.2 percent SDS. Electrophoresis was performed on an LKB Multiphor apparatus with an ISCO power supply. Gels were stained with Coomassie blue R-250. The standard (*STD*) is a sample of partially purified chimeric proinsulin. The numbers indicate the hour of collection.

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interference optics, we estimate that under optimal conditions more than 90 percent of the cells in a culture may contain inclusion bodies. Recent immunocytochemical data have confirmed that these morphologically distinct areas in the cells were sites of localized accumulation of the insulin chain chimeric proteins (*3*).

The proportion of a cell's cytoplasm occupied by the inclusion bodies was dependent on the time that samples were taken. Early in the fermentation, less than 1 percent of the bacterial volume might be occupied by product, whereas later in the fermentation up to 70 percent of a midline longitudinal section of a bacterium could be filled with the inclusion material. In cross section, some cells appeared completely filled with product; and, as illustrated in the scanning electron micrograph (Fig. 2), some cells were filled with sufficient material to cause a bulging of the cell wall. Morphometric analysis (*4*) of electron micrographs of sectioned cells indicated average product levels in a population of bacteria as high as 20 percent of the cell volume.

The chimeric protein aggregates that we have observed during production of recombinant insulin proteins are morphologically distinct from inclusion bodies normally found in bacteria (*5*). Similar aggregates have, however, been reported in *E. coli* grown in the presence of amino acid analogs such as the arginine analog canavanine (*6*, *7*). As has been suggested in the case of canavanine protein aggregation (*7*), the chimeric insulin proteins are relatively insoluble and the formation of inclusions may result from simple precipitation and aggregation within the cell rather than through a mechanistically complex energy dependent process.

The presence of inclusion bodies in *E. coli* that have been genetically altered in order to produce human proteins is an interesting finding that is relevant both to the quantitation of chimeric protein production and to the isolation and purification of the bacterial product for pharmaceutical use. Our results demonstrate that the bacterium is producing significant amounts of material and that the product accumulates in morphologically distinct inclusion bodies in the bacterial cytoplasm. Moreover, the scanning electron micrographs in particular indicate that the inclusion aggregates are sufficiently rigid and cohesive to cause a prominent distention of the bacterial cell wall.

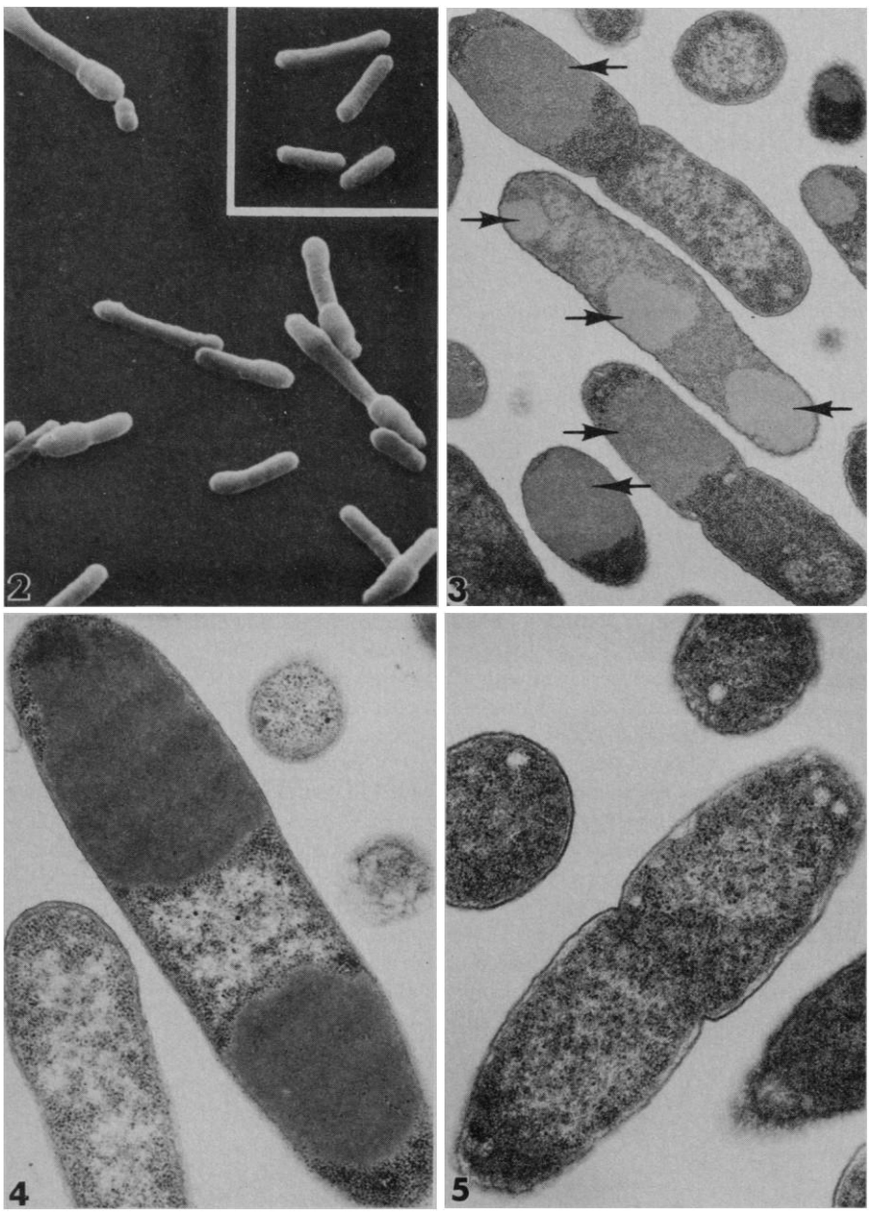

Fig. 2. Scanning electron micrograph of *E. coli* cells fixed in late log phase of growth from a culture producing trp polypeptide-proinsulin chimeric protein. Prominent bulges are evident in the bacterial cell walls. Similar bulges seen in nondried cells by light microscopy corresponded to the presence of intracellular inclusion bodies (×5300). Inset shows cells of the nonplasmid-containing *E. coli* strain for comparison (×5300). Figs. 3 to 5. Transmission electron micrographs. All the cells in these figures were fixed during late log phase of growth (*9*). Fig. 3. Prominent inclusion bodies (arrows) can be seen in several cells of an *E. coli* culture producing β-gal-insulin A chain chimeric protein (×17,500). Fig. 4. Inclusion bodies in *E. coli* cell of culture producing trp polypeptide-proinsulin chimeric protein (×30,000). Fig. 5. Cells of the *E. coli* strain not containing plasmids. Inclusion bodies are not observed in the nonplasmid-containing organisms (×30,000).

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¼-inch or ½-inch point lattice placed over the photographic print. Cell profiles (50 to 100) were counted for each experimental or culture condition being evaluated. The percentage of the cell occupied by inclusion material was estimated by calculating the ratio of points falling on inclusion body material to total points falling on the bacterial cells.
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Crystallization of Recombinant Human Leukocyte Interferon A

Abstract. *Crystals of recombinant human leukocyte interferon A (IFLrA) were prepared, and their composition was characterized. These studies should provide the foundation for determination of the tertiary structure of IFLrA by x-ray crystallography.*

The interferons comprise a family of proteins that activate an antiviral response in animal cells. Apart from their potential use as therapeutic agents, these proteins are of interest because of their multiple effects on cells. Progress has been made in determining the primary structure of both human leukocyte (*1–5*) and fibroblast (*6–9*) interferons despite the limited amounts available. Studies on secondary and tertiary structure have been limited because of the lack of sufficient quantities of pure interferons. Through recombinant DNA technology, moderate amounts of material showing high interferon activity are now available (*10–12*).

Little is known about the fidelity of transcription and translation of mammalian genes in bacteria, or about posttranslational modifications that may occur. It is not certain that bacteria faithfully transcribe and translate all mammalian sequences, nor is it known whether the proteins are substantially modified. Purification of recombinant human leukocyte interferon A revealed that it is homogeneous by electrophoresis and partial amino acid sequencing (*12*); however, a substantial sequence heterogeneity might go undetected. Furthermore, heterogeneity in secondary and tertiary structure remained a real possibility.

The ability of a substance to crystallize in a uniform habit satisfies one of the classical criteria of homogeneity. Crystallization may also permit the removal of trace impurities. Furthermore, when large-ordered single crystals can be obtained, the molecule's tertiary structure may be determined by x-ray crystallography.

Numerous techniques have been developed for the crystallization of proteins (*13*); however, no generalized procedure has been discovered, and many proteins remain uncrystallized. The most widely used approach involves the addition to the protein solution of a crystallizing agent, which is commonly a salt, such as ammonium sulfate or ammonium citrate, or an organic solvent, such as ethanol or 2-methyl-2,4-pentanediol.

One of the most versatile crystallizing agents is polyethylene glycol (PEG), which combines some of the characteristics of the salts and the organic solvents (*14, 15*). Recombinant human leukocyte interferon A (IFLrA) crystallizes readily from PEG, occasionally in large crystal masses.

At low concentrations of IFLrA (0.3 to 1 mg/ml) and high concentrations of PEG-4000 (50 mg/ml) acicular crystals commonly appeared in 1 to 3 days at 4°C (Fig. 1). These crystals were centrifuged, washed with 10 percent PEG-4000, and

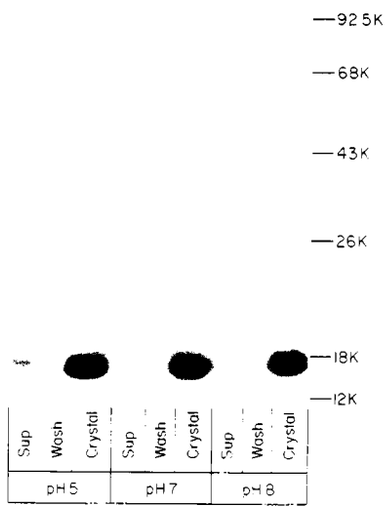

Fig. 1 (left). Crystals of IFLrA, acicular form. IFLrA prepared as previously described was dialyzed against 10 m*M* Hepes (*p*H 7.1) buffer (ammonium salt) and concentrated to 9 mg/ml by centrifugal evaporation. A 20-μl droplet containing IFLrA (2 mg/ml), NH_4-Hepes (50 m*M*, *p*H 7.1), PEG-4000 (30 mg/ml), and NaN_3 (0.5 mg/ml) was placed in the well of a siliconized spot plate and allowed to equilibrate at 4°C by vapor diffusion with PEG-4000 (50 mg/ml). Crystals appeared within 24 hours. The length of the white strip represents 0.1 mm. Fig. 2 (middle). Electrophoretic analysis of crystalline IFLrA. Crystals from a mixture similar to that described in the legend to Fig. 1 were transferred to a 1.5-ml centrifuge tube and washed twice with 100-μl portions of PEG-4000 (100 mg/ml). The crystals were dissolved in 50 μl of H_2O and subjected to polyacrylamide gel electrophoresis. Fig. 3 (right). Crystals of IFLrA, prismatic form. A droplet containing 20 μl of IFLrA (5 mg/ml), NH_4-Hepes (50 m*M*, *p*H 7.1), PEG-4000 (20 mg/ml), and NaN_3 (0.5 mg/ml) was placed in a well of a siliconized spot plate. An amorphous precipitate formed immediately, from which the crystals grew during the following week.

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