Vol. 146, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1987 Pages 232-238

HIGH-PRESSURE INFRARED SPECTROSCOPIC STUDY OF HUMAN PROINSULIN GENE EXPRESSION IN LIVE ESCHERICHIA COLI CELLS

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Received May 16, 1987

SUMMARY: Infrared spectra of *E. coli* strain JM103 and transformants which overproduced recombinant proinsulin have been measured as a function of pressure up to 38 kbar. It is the first time that high-pressure infrared spectra of live bacteria have been successfully measured. In ambient conditions, spectra of the host strain JM103 and the transformants are generally identical. However, under pressure, distinct shifting pattern can be observed in specific spectral parameters of transformants, presumably due to accumulation of proinsulin in form of cytoplasmic inclusion bodies. In particular, the pressure-induced frequency shift of the amide III band (1235 cm^{-1}) in the proinsulin-producing transformants is much smaller than in the host JM103. This pressure effect can potentially be an efficient approach to monitor maximum gene expression in microorganisms. Contrary to predictions based on model system, the pressure-induced denaturation and the sharp transition from disordered liquid crystalline state to the ordered gel state commonly observed in the aqueous solution of protein and aqueous bilayer dispersion of lipids, respectively, do not occur in the bacterial proteins and cell membrane of *E. coli*.

It has been found recently (1) that the rapid degradation of human proinsulin synthesized in *E. coli* by recombinant DNA techniques can be prevented by inserting a DNA sequence encoding a short homooligopeptide, such as $(Ala)_6$, $(Asn)_6$, $(Cys)_7$, $(Gln)_7$, $(His)_6$, $(Ser)_6$ and $(Thr)_6$, at the 5' end of the proinsulin gene. The expressed polypeptide is then accumulated as cytoplasmic inclusion bodies, with the yield of proinsulin ranging between 6% and 26% of the total bacterial protein.

For large scale production of proinsulin, an efficient method for direct determination of maximum gene expression and detection of any biological contamination would be important economically. However, the established procedures of radioimmunoassay and SDS-polyacrylamide gel electrophoresis are time-consuming, and therefore inappropriate as a monitoring process. In a different approach, we have carried out a series of high pressure infrared spectroscopic studies on *E. coli* strain JM103 and the transformants which produce proinsulin fused with either a $(Asn)_6$ or a $(Ala)_6$

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0006-291X/87 $1.50

leader. This was a first attempt to measure infrared spectra of live cells in water as a function of pressure. Spectra of excellent quality have been obtained.

EXPERIMENTAL

The sources of the materials, the method of production of fused proinsulin and the analysis of the proinsulin contained have been described previously (1).

E. coli pellets were obtained after centrifuge from culture medium. The bacterial pellets were placed together with powdered α-quartz in a 0.37 mm diameter hole on a 0.23 mm thick stainless steel gasket mounted on a diamond anvil cell as described previously (2). Pressures on the samples were determined from the 695 cm^{-1} infrared absorption band of quartz (2). Frequencies of this band were obtained from third power derivative spectra, calculated using a breakpoint of 0.3 in the Fourier domain. Pressures were calculated from these frequencies according to the expression $P(kbar)=a_1\Delta\nu+a_2\Delta\nu^2$, where $\Delta\nu$ is the measured frequency shift, $a_1=1.2062$ and $a_2=0.015164$.

Infrared spectra of the samples were measured at 28°C on a Bomem model DA3.02 Fourier transform spectrometer with a liquid nitrogen cooled mercury cadmium telluride detector. The infrared beam was condensed by a sodium chloride lens system onto the sample in the diamond anvil cell. The spectral resolution was 4 cm^{-1}, and typically 1000 scans were co-added for each spectrum. Data reduction were performed using software developed in this laboratory.

RESULTS AND DISCUSSION

Figure 1 shows the infrared spectra of *E. coli* strain JM103 (A) and two transformants containing aggregates of proinsulins fused with either a $(Ala)_6$ (B) or a $(Asn)_6$ (C) leader. The corresponding resolution-enhanced infrared spectra have been obtained by third power derivative using a break-

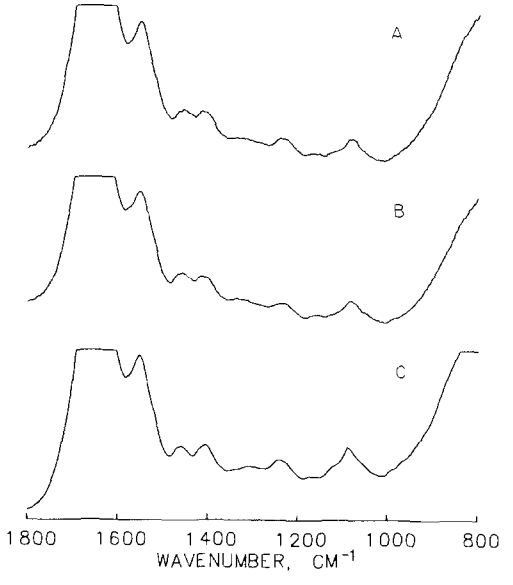

Fig. 1. Infrared spectra of *E. coli* JM103 (A) and transformed cells containing proinsulin fused with $(Ala)_6$ (B) or $(Asn)_6$ (C) leader.

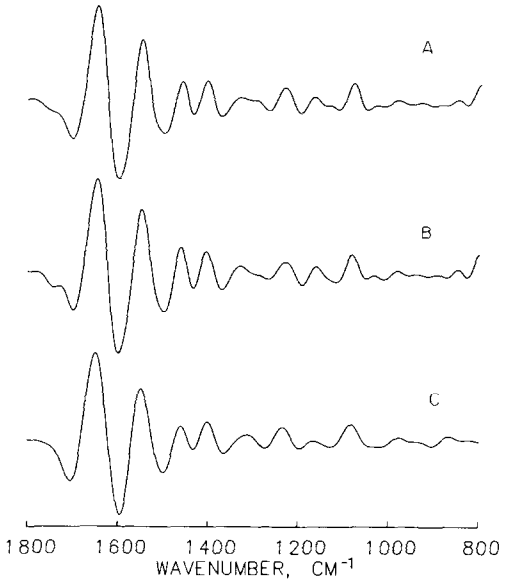

Fig. 2. Resolution-enhanced derivative infrared spectra of *E. coli* JM103 cells (A) and transformed *E. coli* containing proinsulin fused with $(Ala)_6$ (B) and $(Asn)_6$ (C) leaders.

point of 0.1 in the Fourier domain (3) (Fig. 2). Most of the infrared bands in the frequency region shown in Figures 1 and 2 are due to the vibration modes of the skeletal amide groups and the side chain groups of the bacterial proteins with the exception of the band at 1664 cm^{-1} which is due to the bending mode of water molecules. It is clear from Figures 1 and 2 that the main features in the infrared spectra of the proinsulin-producing transformants are generally identical to that of the *E. coli* host strain, although subtle differences in the infrared spectra can still be observed among these samples. However, the effect of pressure on specific spectral parameters of the proinsulin-producing transformants is quite different from that of the *E. coli* host JM103. The pressure-induced frequency shift of the amide III band (1235 cm^{-1}) of proinsulin-producing transformants is much smaller than that of the *E. coli* host JM103 (Fig. 3). This pressure shift of the amide III band frequency is completely reversible in all the three *E. coli* samples (Fig. 3; only the reversion results of *E. coli* host JM103 are shown).

The amide III band in proteins is mainly due to the C-N-H in-plane bending vibration and its location in the infrared spectrum is determined by the conformational structure of protein molecules (4). For a β-sheet structure this band is generally found in the frequency region 1220-1240 cm^{-1}. Therefore, the band at 1235 cm^{-1} observed in all *E. coli* samples at ambient conditions are due to the amide III band of the β-sheet portions of the cellular proteins. Frequency of the same band is affected by the strength of the hydrogen bond on the NH group (5). Obviously, the hydrogen bonds in

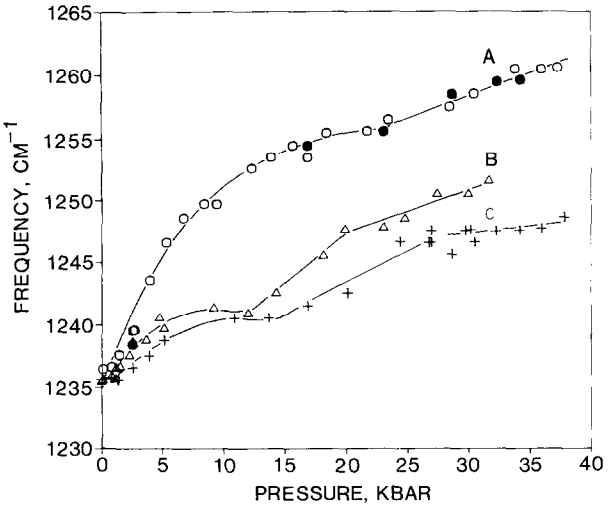

Fig. 3. Pressure dependences of the Amide III band frequencies of *E. coli* JM103 cells (A) and transformed cells containing proinsulin fused with $(Asn)_6$ (B) and $(Ala)_6$ (C) leaders. Full circles were obtained by reducing pressure.

the bacterial proteins of the *E. coli* JM103 and the proinsulin-producing transformants responds differently to identical external pressure as demonstrated by variation of the frequency of the amide III band (Fig. 3).

The large difference in the pressure shift of the amide III band, between the *E. coli* cells and the transformants overproducing proinsulin thus provides an efficient technique for on-line monitoring of the production of recombinant proteins in *E. coli*. A decrease in this frequency from 1250 cm^{-1} to 1240 cm^{-1} at 10 kbar, which can be measured within 10 minutes, indicates that the genes in the *E. coli* sample have reached the maximum expression and the recombinant protein has been accumulated as aggregates.

Two discontinuities in the pressure dependence of the amide III frequency marked as changes of slope are observed (Fig. 3). The first one is at about 10 kbar for all three *E. coli* samples. The second one is in the pressure range 21-29 kbar. The appearance of these discontinuities indicates that some structural changes in the protein molecules take place in the *E. coli* samples at these pressures. These structural changes are certainly not originated from the secondary structure of the protein molecules since the location of the amide III band is quite different among the various conformational structures of protein molecules (4). They are most likely the results of the changes in the packing within the hydrogen bond network of the protein molecules in the cells. These pressure-induced structural changes are reversible as indicated by the reversible frequency shift of the amide III band.

Pressure-induced changes in the molecular packing of the proteins in *E. coli* cells are further evident from the pressure effect on the

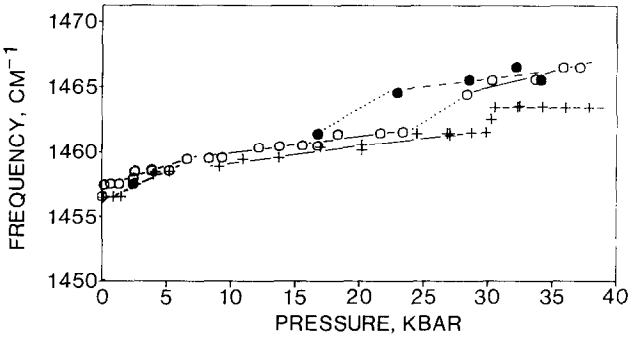

Fig. 4. Pressure dependences of the frequencies of the methyl bending mode of the *E. coli* JM103 (O) and the *E. coli* containing $(Ala)_6$-fused proinsulin (+). Full circles were obtained by reduced pressure.

frequency of the 1457 cm^{-1} band of *E. coli* JM103 and proinsulin-producing transformants (Fig. 4; results of a proinsulin-producing transformant are shown). This band is due to the asymmetric bending mode of the methyl groups in the side chains of the protein backbone (6). The pressure-induced frequency shift of this band is much smaller than that of the amide III and other amide (not shown) bands. The two discontinuities in the pressure dependences of the amide III and other (not shown) frequencies are also observed in the pressure dependence of the 1457 cm^{-1} band frequency at the corresponding pressures. The pressure effect of this frequency is also reversible and exhibits a pressure hysteresis in the second discontinuity at 21-29 kbar (Fig. 4; only the hysteresis of *E. coli* JM103 are shown). Since the 1457 cm^{-1} band is due to the methyl bending mode of the methyl groups in the side chains, its frequency is not affected by the conformational structure in the protein backbone, and thus the pressure-induced changes in the frequency of this band is solely the result of changes in the interactions of the methyl groups with the neighboring functional groups within or between the protein molecules in the cells. Consequently, the discontinuities in the pressure dependence of this frequency indicate that the linear pressure effect on the interactions of the methyl side groups is interrupted at these discontinuities as a result of changes in the molecular packing. The changes in the packing of protein molecules relax completely as pressure is released. However, the relaxation of the second packing-change in the pressure range 21-29 kbar is much more sluggish than the first one at 10 kbar for all the *E. coli* samples which is shown by the pressure hysteresis of the frequency shift at the second transition (Fig. 4; only the hysteresis of *E. coli* JM103 is shown). This changes in the molecular packing of proteins are presumably arising from the pressure-induced volume change of the internal cavities (7).

It is well known that pressure usually induces an irreversible denaturation of proteins in the pressure range of 5 kbar (8). This pressure-

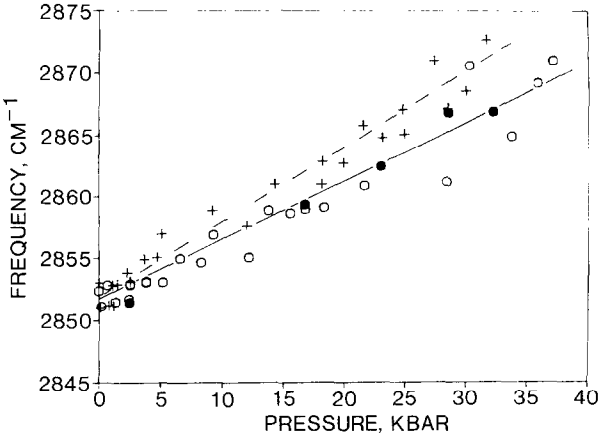

Fig. 5. Pressure dependences of the frequencies of the methylene CH_2 symmetric stretching mode of *E. coli* JM103 (O) and the transformants containing $(Ala)_6$-fused proinsulin (+). Full circles were obtained by reduced pressure.

induced denaturation results in a permanent change in the secondary structure of protein molecules. It is interesting that no such irreversible denaturation of protein molecules is induced by pressure in *E. coli* cells. Pressure can only induce some reversible changes in molecular packing of proteins in the cells.

Figure 5 shows the pressure dependences of the frequencies of the 2852 cm^{-1} band for *E. coli* JM103 and transformants which produced fused proinsulines (of the two proinsulin-producing transformants, only the results of the *E. coli* with $(Ala)_6$-proinsulin are shown in Fig. 5). This band is due to the symmetric stretching of the methylene groups of the lipid molecules in the cell membranes. The frequency of this band at ambient temperature and pressure is at 2852 cm^{-1} for all *E. coli* samples, which is the characteristic frequency for biomembrane lipid in the liquid crystalline state (9). The pressure shift of this band in the transformants containing $(Ala)_6$-fused proinsulin is slightly larger than that in *E. coli* JM103 sample. This suggests that the packing of the lipids of cellular membrane is slightly tighter in the transformants bulging with the recombinant protein. In model biomembranes, a transition from the liquid crystalline phase to the gel phase is usually induced by external pressure and the frequency of the CH_2 stretching band of the membrane lipids decreases sharply at the transition pressure (9-11). This discontinuity is not observed in the *E. coli* samples (Fig. 5) and thus the pressure-induced sharp transition from the liquid crystalline phase to the gel phase in the model biomembrane systems does not actually occur in the biomembranes of *E. coli* bacteria. Presumably the pressure-induced conformational ordering of the lipid chains in the cell membranes of *E. coli* does not take place.

ACKNOWLEDGMENTS

Issued as NRCC Publication No. 27591. We would like to thank Dr. T.E. Chagwedera for recording some preliminary infrared spectra.

REFERENCES

1. Sung, W.L., Yao, F.-L., Zahab, D.M. and Narang, S.A. (1986) Proc. Natl. Acad. Sci. USA 83, 561-565.
2. Wong, P.T.T., Moffatt, D.J. and Baudais, F.L. (1985) Appl. Spectrosc. 39, 733-735.
3. Kauppinen, J.K., Moffatt, D.J., Mantsch, H.H. and Cameron, D.G. (1981) Anal. Chem. 53, 1454-1457.
4. Koenig, J.L. (1979) NATO Adv. Study Inst. Series, Series C. 43, 109-124.
5. Ambrose, E.J. and Elliott, A. (1951) Proc. Roy. Soc. A. 205, 75-90.
6. Dwivedi, A.M. and Krimm, S. (1984) Biopolymer 23, 923-943.
7. Rashin, A.A., Iofin, M. and Honig, B. (1986) Biochemistry 25, 3619-3625.
8. Heremems, K. and Wong, P.T.T. (1985) Chem. Phys. Lett. 118, 101-104.
9. Wong, P.T.T., Murphy, W.F. and Mantsch, H.H., (1982) J. Chem. Phys. 76, 5230-5237.
10. Siminovitch, D.J., Wong, P.T.T. and Mantsch, H.H. (1987) Biochemistry, in press.
11. Wong, P.T.T. and Mantsch, H.H. (1986) J. Raman Spectrosc. 17, 335-340.