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# Infrared spectroscopic discrimination between $\alpha$ - and $3_{10}$ -helices in globular proteins

Reexamination of Amide I infrared bands of  $\alpha$ -lactalbumin and their assignment to secondary structures

STEVEN J. PRESTRELSKI, D. MICHAEL BYLER\* and MARVIN P. THOMPSON

USDA, ARS, Eastern Regional Research Center, Philadelphia, Pennsylvania, USA

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We have undertaken a new and more detailed Fourier-transform infrared (FTIR) spectroscopic study of  $\alpha$ -lactalbumin (in  $D_2O$  solution) aimed at correlating its secondary structures to observed Amide I' infrared bands. The spectra reported here were interpreted in light of the recently determined crystal structure of  $\alpha$ -lactalbumin and by comparison with the spectra and structure of the homologous protein lysozyme. Of particular importance is the new evidence supporting the assignment of the band at  $1639\text{ cm}^{-1}$  to  $3_{10}$ -helices. This assignment is in excellent agreement with one based on theoretical and experimental studies of  $3_{10}$ -helical polypeptides. The frequency observed for  $3_{10}$ -helices is distinctly different from that at which  $\alpha$ -helices are typically found (viz., around  $1655\text{ cm}^{-1}$ ). In the present study, two bands are clearly resolved in the latter region at  $1651$  and  $1659\text{ cm}^{-1}$ . Both are apparently associated with  $\alpha$ -helices. These results suggest that for  $D_2O$  solutions of globular proteins, FTIR spectroscopy can be a facile method for detecting the presence of these two different types of helical conformation and distinguishing between them. This provides a distinct advantage over ultraviolet circular dichroism spectroscopy (UV-CD). This work also provides a basis for future studies of  $\alpha$ -lactalbumin which examine the effects of environment (e.g., pH, temperature) and ligands (e.g.,  $Ca^{2+}$ ,  $Mn^{2+}$ ) on its conformation.

*Key words:* deconvolution; helical structure; infrared spectroscopy;  $\alpha$ -lactalbumin; lysozyme; protein secondary structure

$\alpha$ -Lactalbumin, a major  $Ca^{2+}$ -binding whey protein in the milk of most species of mammals, has been described as the modifier protein in lactose synthesis. It complexes with galactosyl transferase altering the substrate specificity of the enzyme to favor glucose as the acceptor molecule (1). In addition to its importance in physiological systems,  $\alpha$ -lactalbumin has been shown to exhibit potential antitumor activity in human mammary carcinoma cell lines (2). Thus, it is of importance to be able to determine the structure of  $\alpha$ -lactalbumin in a variety of functionally relevant environments.

Fourier-transform infrared (FTIR) spectroscopy is a valuable tool for the study of protein conformation, in diverse environments, by virtue of the conformation-sensitive amide bands (3–5). Of these, the Amide I band ( $1700\text{--}1620\text{ cm}^{-1}$ ), which arises mainly from

backbone  $C=O$  stretching vibrations, is the best understood and has been found generally to be the most useful for monitoring conformational changes. This vibrational mode has been shown to be sensitive to small variations in molecular geometry and hydrogen bonding (6) and, therefore, different patterns of folding of the peptide backbone give rise to bands at discrete frequencies. Unfortunately, the characteristic Amide I band components of proteins in aqueous environments are typically broad and their band centers fall close together in frequency. The substantial overlap that results frequently causes the observed absorption to appear almost featureless. In such circumstances increased instrumental resolution is of no avail. Instead, mathematical procedures, termed resolution-enhancement or band narrowing techniques, are required to resolve this absorption into its component bands. These techniques do nothing to affect the instrumental resolution, but when applied to unresolved absorptions, the resulting mathematical

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Abbreviations: CD, circular dichroism; FTIR, Fourier-transform infrared; IR, infrared.

modification of the data enhances the spectroscopist's ability to visualize overlapped bands. Derivative spectroscopy and Fourier self-deconvolution are two such resolution-enhancement methods which have been successfully applied to the analysis of protein infrared spectra (3–5, 7).

Completely successful infrared spectroscopic analysis of secondary structure of proteins necessitates an unambiguous knowledge of the correspondence between different folding types and individual component bands in the Amide I region. Analysis of spectra in the light of crystallographically determined structures can aid the process of spectra-structure correlation (3, 8). Several systematic studies of the IR spectra of proteins in D<sub>2</sub>O solutions using band-narrowing procedures have been reported (see ref. 4 for a review). (In D<sub>2</sub>O, exchange of backbone hydrogens for deuterium atoms shifts the Amide I vibrational frequencies 5–15 cm<sup>-1</sup>; these are designated as Amide I'.) Such investigations have correlated Amide I' infrared data with protein structures determined from X-ray crystallography. These studies have produced band assignments of  $\alpha$ -helix, extended strand, reverse turn, and orderless structures.

The crystal structure of  $\alpha$ -lactalbumin has been recently determined at 1.7 Å atomic resolution (9). The high degree of sequence homology between  $\alpha$ -lactalbumin and hen egg white lysozyme (10) was found to result in almost identical three-dimensional structures. In this report we present the spectrum of  $\alpha$ -lactalbumin in the presence of 20 mM Ca<sup>2+</sup>. The assignments reported here are based on a comparison with the spectrum of lysozyme. Of particular interest is the new assignment of the band near 1639 cm<sup>-1</sup> to 3<sub>10</sub>-helices. Our studies are part of a continuing project to produce a reliable data base for correlating the Amide I' infrared spectra and X-ray structures across a broad range of globular proteins.

## EXPERIMENTAL PROCEDURES

### Materials

$\alpha$ -Lactalbumin (Type I, from bovine milk) and lysozyme (Grade IV, from hen egg white) were purchased from Sigma. The purity of  $\alpha$ -lactalbumin was determined electrophoretically using SDS-PAGE and non-denaturing PAGE. All other chemicals used were reagent grade.

### Infrared spectra

**Sample preparations.** For IR spectroscopy proteins were prepared as 3.5% (w/v) solutions in 20 mM imidazole buffers (pD = 6.9) made with D<sub>2</sub>O. pD was determined by adding 0.4 to the pH measured with a glass electrode (11). For  $\alpha$ -lactalbumin, sufficient 1 M CaCl<sub>2</sub> in D<sub>2</sub>O was added to make the final solution 20 mM in Ca<sup>2+</sup>. Solutions were placed in 75  $\mu$ m path-length CaF<sub>2</sub> IR cells with teflon spacers.

**Instrumentation.** Spectra were collected at ambient temperature using a Nicolet 740 SX FTIR system equipped with a water-cooled Globar source, a Ge-coated KBr beam splitter and a broad-range mercury-cadmium-telluride detector at a nominal resolution of 2 cm<sup>-1</sup> by collecting 4096 coadded, double-sided interferograms (0.44 s/scan) which were Fourier-transformed after using a Happ-Genzel apodization function. The spectrometer and sample chamber were purged continuously with dry nitrogen gas. Spectral contributions from residual H<sub>2</sub>O vapor in the light path and from buffers were subtracted using programs provided with the Nicolet FTIR software, Version 4.3.

### Resolution-enhancement and curve-fitting procedures

Differentiation and deconvolution of the observed spectra were performed using the Nicolet FTIR Software, Version 4.3, as described previously (3, 7), except that second derivative spectra (Fig. 1) were obtained by applying DR1, the first derivative function, twice. The deconvolution algorithm is based on the method of Kauppinen (12). Values of 18 cm<sup>-1</sup> and 2.8 were used for the undeconvoluted halfwidth and the resolution-enhancement factor, respectively. A Bessel apodization function was used. Curve-fitting of

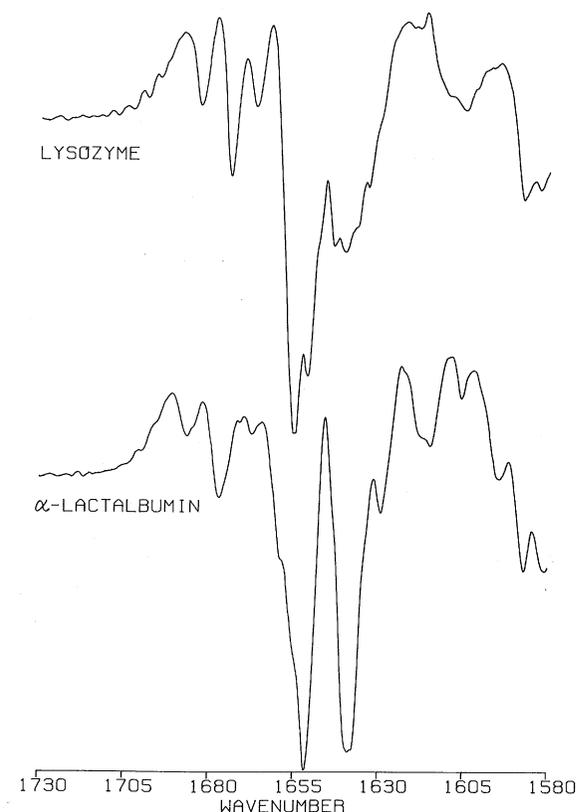
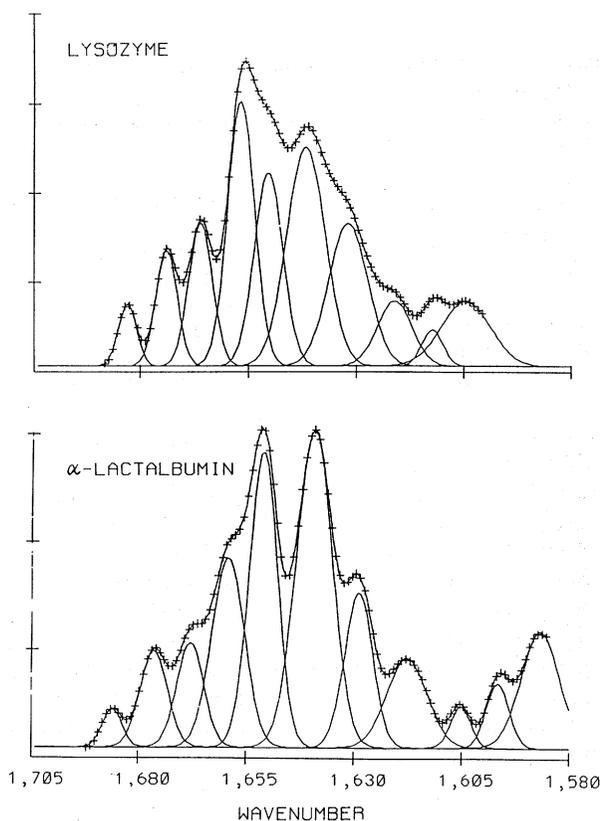


FIGURE 1

Unsmoothed second derivative spectra of  $\alpha$ -lactalbumin and lysozyme (3.5% in 20 mM imidazole buffer, pD = 6.9). The spectrum of  $\alpha$ -lactalbumin was recorded in 20 mM Ca<sup>2+</sup>.



**FIGURE 2**  
Deconvoluted spectra of  $\alpha$ -lactalbumin and lysozyme (+ + + +) (Each cross represents one data point). Individual Gaussian Bands (---) and their sum (—). (Note that the solid line representing the sum of the calculated Gaussian components fits the observed data (+ + + +) with negligible deviation.) Peaks in these spectra below  $1620\text{ cm}^{-1}$  are not Amide I' components but are most likely due to side chain absorbances (19). These additional peaks are included in the curve-fitting procedure to avoid the approximation otherwise incurred with the addition of a sloping baseline parameter.

the deconvoluted spectra (Fig. 2) was performed using an iterative, Gauss-Newton non-linear regression algorithm developed at our laboratory (3). Peaks below  $1620\text{ cm}^{-1}$  are not Amide I bands but their inclusion in the curve-fitting procedure precludes the necessity of assuming a sloping baseline correction. Initial frequencies were taken from the measured frequencies of the resolution-enhanced spectra (Figs. 1–2). Initial band heights and widths were estimated visually. Then all parameters (frequency, height, and width) of all nine bands were allowed to iterate simultaneously until convergence to the best fit was achieved. This occurs when the change between iteration  $n$  and  $n + 1$  in the sum of squares of the deviations between the observed and calculated absorbances is less than a predetermined value.

## RESULTS AND DISCUSSION

FTIR spectra of  $\alpha$ -lactalbumin and lysozyme have been reported previously (3). However, the published

spectrum of  $\alpha$ -lactalbumin, a strong  $\text{Ca}^{2+}$ -binding protein, was recorded for the calcium-depleted form while the spectrum used in this study is of  $\alpha$ -LA in the presence of  $20\text{ mM CA}^{2+}$ . This facilitates comparison with the recently published crystal structure (9) which has been determined with calcium bound to the protein. Additionally, the spectra reported here for both proteins have a higher signal-to-noise ratio than the previous spectra. This has resulted in improved resolution-enhancement which has disclosed bands not previously observed. Comparison of these spectra with those previously reported reveals significant differences. Part of the differences between the old and new spectra of  $\alpha$ -LA can be attributed to the presence or absence of bound calcium.

Fig. 1 shows the second derivative and Fig. 2 shows the deconvoluted, curve-fitted spectra of  $\alpha$ -lactalbumin and lysozyme. Table 1 lists the positions of the Amide I' component peaks and their intensity (integrated band area) relative to the total Amide I' area determined by curve-fitting. As expected, the high degree of correspondence in the secondary and tertiary structures of  $\alpha$ -lactalbumin and lysozyme results in infrared spectra with rather similar Amide-I' profiles. Resolution-enhanced spectra of each of these proteins display the same number of Amide I' component bands with nearly the same frequency positions and roughly comparable relative integrated intensities, with the exception of the two bands near  $1651$  and  $1658\text{ cm}^{-1}$ . While differences are observed, the overall Amide I' envelope of the second derivative spectra of both proteins resemble each other; the two bands with the greatest peak intensity in both spectra occur near  $1640$  and  $1655\text{ cm}^{-1}$  (Fig. 1). The most easily noted distinction in the deconvoluted spectra of the two proteins is the reversal of the relative integrated intensities of the two bands near  $1651$  and  $1658\text{ cm}^{-1}$  (Fig. 2).

The crystal structure of  $\alpha$ -lactalbumin reported by Acharya *et al.* (9) reveals that 31% of the residues in  $\alpha$ -lactalbumin adopt the  $\alpha$ -helical conformation. Bands in the range from  $1650$  to  $1659\text{ cm}^{-1}$  in infrared spectra of deuterated proteins have been assigned to  $\alpha$ -helices (3, 4, 7).  $\alpha$ -Lactalbumin and lysozyme each have two bands in this region, at approximately  $1651$  and  $1658\text{ cm}^{-1}$ . Although their individual intensities differ, the sum of the relative intensities of this pair of bands totals 0.40 for  $\alpha$ -lactalbumin and 0.37 for lysozyme. In the spectra of both proteins, both bands fall within the range of frequencies found for  $\alpha$ -helix absorption (3, 4, 7). These discrete bands do not arise from incomplete deuteration of the peptide NH groups of helices. If this were the case, examination of the spectra of the two proteins would reveal evidence of NH stretching bands (Amide A) and CNH deformation bands (Amide II) around  $3300$  and  $1550\text{ cm}^{-1}$ , respectively, due to unexchanged peptide residues. (Upon deuteration, the Amide A and Amide II vibrational modes undergo large shifts to lower frequency.)

TABLE 1

Peak positions and relative integrated intensities of the Amide I' regions of  $\alpha$ -lactalbumin and lysozyme calculated from curve-fitted, deconvoluted spectra (Fig. 2)

$\alpha$ -Lactalbumin		Lysozyme		Assignment (3, 4, 7)
$\nu$ ( $\text{cm}^{-1}$ )	A	$\nu$ ( $\text{cm}^{-1}$ )	A	
1629	0.12	1632	0.17	Extended strand ( $\mathbf{B}_1$ )
1639	0.31	1642	0.25	$3_{10}$ -Helix
1651	0.23	1650	0.16	$\alpha$ -Helix
1659	0.17	1657	0.21	$\alpha$ -Helix
1668	0.08	1666	0.10	Turns
1676	0.07	1674	0.07	Extended strand ( $\mathbf{A}_1$ )/turns
1686	0.02	1684	0.04	Turns

Nor does it seem likely that these bands represent the  $\mathbf{A}$  and  $\mathbf{E}_1$  components predicted for  $\alpha$ -helices from theoretical studies (6) and recently reported to be observed in the spectrum of hemoglobin at  $1\text{ cm}^{-1}$  resolution (14). If this were true, the intensity of the low frequency band should be greater than that of the high frequency mode in the spectra of lysozyme as well as that of  $\alpha$ -lactalbumin. In addition, the  $7\text{--}8\text{ cm}^{-1}$  splitting observed between these bands in the present study is larger than the  $2\text{--}3\text{ cm}^{-1}$  difference in the  $\mathbf{A}$  and  $\mathbf{E}_1$  Amide I vibrational modes of  $\alpha$ -helices reported by previous investigators for both observed (6, 14) and calculated values (6). Instead, the presence of two  $\alpha$ -helical components in the spectra of  $\alpha$ -lactalbumin and lysozyme appears to result from small differences in hydrogen bonding or geometry among the individual helical segments. Apparently, a single protein may occasionally have two distinct bands associated with its  $\alpha$ -helices.

$\alpha$ -Lactalbumin and lysozyme are somewhat unusual in that they both contain a relatively high amount of a second type of helix, termed  $3_{10}$ -helix. This conformation accounts for approximately 20% of the secondary structure in each of these two proteins (9). The  $3_{10}$ -helix differs from the  $\alpha$ -helix in that the latter has intramolecular hydrogen bonds between the peptide carbonyl oxygen of residue  $i$  and the NH group of residue  $i + 4$ , while the former helix is hydrogen bonded between residues  $i$  and  $i + 3$ . The  $3_{10}$ -helix appears infrequently in globular proteins; when it does exist, it is typically as a final turn of an  $\alpha$ -helix (13). However, in the  $\alpha$ -lactalbumin and lysozyme structures, the  $3_{10}$ -helices occur in relatively long stretches and are not necessarily contiguous with  $\alpha$ -helices. In a recent study of cytochrome  $b_5$ , another protein with  $\sim 30\%$   $3_{10}$ -helix, Holloway & Mantsch (15) have suggested that this type of helix absorbs around  $1639\text{ cm}^{-1}$ . Previously, most investigators (3–5, 7) had assigned all peaks in the  $1640\text{--}1620\text{ cm}^{-1}$  region as the low frequency components ( $\mathbf{B}_1$ ) of extended strand vibrations, although of the proteins they examined, few with a large fraction of extended structures exhibited absorbances as high as  $1639\text{ cm}^{-1}$ .

Given the high relative intensity of the peak around  $1640\text{ cm}^{-1}$  in the spectrum of both  $\alpha$ -lactalbumin and lysozyme and the low fraction of the peptide chain in each which adopts the extended strand conformation, the assignment of these bands near  $1640\text{ cm}^{-1}$  to  $3_{10}$ -helix is certainly more compatible with the results from the X-ray diffraction (9). Additionally, both proteins exhibit other peaks at about  $1630\text{ cm}^{-1}$  whose frequency and relative intensities are more consistent with the amount of extended structure reported in these two proteins. Thus, these new IR data on  $\alpha$ -lactalbumin and lysozyme corroborate the assignment first suggested by Holloway & Mantsch (15) that, for proteins in  $\text{D}_2\text{O}$ ,  $3_{10}$ -helices absorb around  $1639\text{--}1640\text{ cm}^{-1}$ , about  $10\text{--}15\text{ cm}^{-1}$  lower in frequency than do  $\alpha$ -helices. These authors also note that such a frequency difference would be expected because the former helix is a tighter one than the latter.

The assignment of bands near  $1640\text{ cm}^{-1}$  in the spectra of  $\alpha$ -lactalbumin and lysozyme in  $\text{D}_2\text{O}$  solutions to  $3_{10}$ -helices is further supported by vibrational studies of poly-( $\alpha$ -aminoisobutyric acid), which reportedly adopts a  $3_{10}$ -helical conformation (16). The observed Amide I' frequency for the N-deuterated derivative of this polypeptide was  $1640\text{ cm}^{-1}$  for both the infrared and Raman experiment. This study also noted the significantly larger frequency shift observed on deuteration of  $3_{10}$ -helices compared to that of  $\alpha$ -helices. In  $\text{H}_2\text{O}$ , both the  $\alpha$ - and  $3_{10}$ -helices are predicted to show similar Amide I frequencies (6). However, the much larger shift observed on deuteration of  $3_{10}$ -helices ( $16\text{ cm}^{-1}$  compared to  $5\text{ cm}^{-1}$  for  $\alpha$ -helices) results in a large frequency separation of the two helices, thus making them distinguishable. This behavior also argues in favor of performing infrared studies of protein conformation in  $\text{D}_2\text{O}$  rather than  $\text{H}_2\text{O}$ .

This ability of FTIR to distinguish readily between  $3_{10}$ - and  $\alpha$ -helices is a clear advantage over ultraviolet CD spectroscopy. A recent review of Johnson (17) concerning protein secondary structure estimation using this technique gives no indication that it can discriminate between these two types of helices.

$\alpha$ -Lactalbumin has a full complement of reverse turns representing Type I, II, III, and III'  $\beta$ -turns, as well as other types of turns (9, 10). The IR spectra of both  $\alpha$ -lactalbumin and lysozyme exhibit peaks about 1667, 1675, and 1685  $\text{cm}^{-1}$  which accords with the presence of reverse turns, although the high frequency ( $\mathbf{A}_1$ ) component of extended strand vibrations also falls in the same region as the latter two bands (3, 4). Although theoretical and experimental studies of model peptides (7) have revealed that different classes of turns exhibit different infrared frequencies, to date, no unequivocal correlation has been proposed between specific classes of reverse turns in proteins and individual bands in the Amide I' region.

Conspicuously absent from the spectra of both  $\alpha$ -lactalbumin and lysozyme is any peak near 1645  $\text{cm}^{-1}$ , a region which has been assigned to unordered conformations in proteins in  $\text{D}_2\text{O}$  (3, 4). The unsmoothed second derivative spectrum of lysozyme appears to have a very small feature at 1643  $\text{cm}^{-1}$ , but no peak at this frequency is apparent in the deconvoluted spectrum, nor is such a component necessary to achieve a satisfactory fit of the deconvoluted spectrum. Thus, even if such a component is truly present and not just noise or a residual, incompletely subtracted water vapor line, its intensity is negligible relative to the total Amide I' area. The absence of a band around 1645  $\text{cm}^{-1}$  for  $\alpha$ -lactalbumin is consistent with its crystal structure:  $\alpha$ -lactalbumin contains little or no structure which can be described as unordered or irregular. Indeed, on the basis of their crystallographic data, Acharya *et al.* (9) fail to assign only 17 of the 123 residues in  $\alpha$ -LA to some class of regular secondary structure; however, Levitt & Greer (18) assign several homologous residues in lysozyme to the extended strand conformation. The remainder occur as stretches of one of three residues. These data imply that, although on the basis of X-ray data certain residues cannot be assigned with any confidence to a specific class of regular secondary structure, they do possess some degree of order.

#### CONCLUSION

These spectra of  $\alpha$ -lactalbumin and lysozyme provide new and more satisfactory evidence that resolution-enhanced FTIR spectroscopy (in contrast to UV-CD spectroscopy) can distinguish  $3_{10}$ -helices from  $\alpha$ -helices. In  $\text{D}_2\text{O}$  solution, the latter absorb around 1655  $\text{cm}^{-1}$  while the former give bands near 1640  $\text{cm}^{-1}$ . The Amide I' frequencies for these two conformations in proteins agree very well with theoretical predictions and experimental observations of  $\alpha$ - and  $3_{10}$ -helical polypeptides. The new assignment of components in the range of 1638 to 1642  $\text{cm}^{-1}$  to  $3_{10}$ -helices suggests that the upper limit for the frequency range of the low frequency ( $\mathbf{B}_1$ ) component of extended strand structures may need to be revised

somewhat downward, from the region around 1640  $\text{cm}^{-1}$  to about 1637  $\text{cm}^{-1}$ . These new findings should prove useful in future infrared spectroscopic studies of  $\alpha$ -lactalbumin and lysozyme which seek to probe the effects of temperature, pH, ions and other ligands on the conformation on these proteins.

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Addresses:

Dr. D. Michael Byler  
Department of Chemistry and Physical Science  
Philadelphia College of Textiles and Science  
School House Lane and Henry Avenue  
Philadelphia, PA 19144-5497, USA

Dr. Steven J. Prestrelski  
AMGEN, Incorporated  
1840 Dehavilland Drive  
Thousand Oaks, CA 91320, USA

Dr. Marvin P. Thompson  
1432 Manor Lane  
Blue Bell, PA 19422-2022, USA