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EXAMINATION OF SLOW, REVERSIBLE VARIATIONS IN PROTEIN  
SECONDARY STRUCTURE BY FTIR SPECTROSCOPY

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Fourier-transform infrared [FTIR] spectroscopy (augmented by Fourier deconvolution, second derivatives [DR2], and subtraction techniques) affords a powerful means of detecting and assessing spectral differences arising from small variations in structure at the submolecular level. For example, the stretching vibrations of hydrogen-bonded carbonyls (C=O) of the peptide backbone of a protein couple to produce a cluster of absorptions in the amide I region (1700-1620  $\text{cm}^{-1}$ ). The frequency, intensity, width, and shape of each band component is a sensitive function of bonding and geometry of the absorbing group. Ideally, each band may be correlated with specific secondary structures, such as  $\alpha$ -helix,  $\beta$ -sheet, turns, and disordered regions [1-3]. Due to extensive overlap, the normal FTIR spectra of proteins typically have a single, undifferentiated, broad amide I band. By contrast, DR2-FTIR spectra of proteins often disclose a wealth of fine detail, with many of the individual amide I components resolved [1-4]. Hence, DR2-FTIR spectroscopy has great potential as a diagnostic probe of subtle changes in protein backbone conformation that may be induced by environmental perturbations, such as variations in pH and temperature.

To illustrate the utility of this methodology, this paper presents a reexamination of the slow, reversible change in the conformation of the whey protein  $\beta$ -lactoglobulin [ $\beta$ LG] under mildly alkaline solutions. Tanford et al. [5] identified this transition 30 years ago by optical rotation measurements. At ambient temperature and pressure and a pH between about 7 to 8.5,  $\beta$ LG in aqueous solution is presumed to undergo a reversible dissociation (dimer  $\rightleftharpoons$  monomer) [6]. Optical rotatory dispersion [ORD] data indicate that modifications in the backbone conformation accompany this change in quaternary structure [6]. But the exact nature of the structural changes which occur have not been completely deciphered.

In this note we will discuss our DR2-FTIR results on  $\beta$ LG as a function of time at two pH's on either side of the Tanford transition: 6.2 and 7.8. At the lower pH, the conformational structure of the protein is found to be stable and does not vary with time. At the more alkaline pH, we observed small, but distinct, alterations in the secondary structure of the protein over a period of days.

EXPERIMENTAL

$\beta$ LG AB from bovine milk (Cat. #L0130) was obtained from Sigma Chemical Co. [7]. The protein was dissolved (at 0.5% or 3.0% w/v) in a 0.05M phosphate buffer made up in  $\text{D}_2\text{O}$  (at pD 6.2 or 7.8).

FTIR spectra ( $2 \text{ cm}^{-1}$  resolution,  $0.44 \text{ s/scan}$ ) were collected at ambient temperature on a Nicolet 740 FTIR system (equipped with a water-cooled Globar source, a Ge-coated KBr beamsplitter, a broad-range MCT detector) as 4096 co-added double-sided interferograms in a  $75 \mu\text{m}$  pathlength  $\text{CaF}_2$  IR cell [3].

### RESULTS AND DISCUSSION

Examination of four DR2-FTIR spectra of 3% (w/v)  $\beta\text{LG}$  at pD 6.2, collected at various times after dissolution (Fig. 1), shows no appreciable change in the relative intensities and widths of the Amide I band components, even after the protein was incubated at room temperature for nearly ten days. Small, monotonic, time-dependent decreases in the Amide I band frequencies (none more than  $1.7 \text{ cm}^{-1}$  total) probably result from isotopic shifts that accompany the deuteration of a few "hard-to-exchange" peptide protons. Spectra of 0.5% (w/v) protein solutions are quite similar.

These observations indicate that the secondary structure of  $\beta\text{LG}$  is in a stable state at pD 6.2. Previous studies [1-4] have shown that the bands at  $1622$ ,  $1634$ , and  $1678 \text{ cm}^{-1}$  are probably associated with the nine  $\beta$ -strands (51%) observed in the x-ray diffraction study of the crystalline protein [8]. (The percentages given in parentheses represent the fraction of the 162 peptide residues of  $\beta\text{LG}$  identified in the X-ray study as belonging to a particular type of structure.) The  $1649 \text{ cm}^{-1}$  band most likely is an unresolved composite of bands associated with the 11  $\alpha$ -helix residues (7%) and disordered structures (30%). The  $1665 \text{ cm}^{-1}$  band

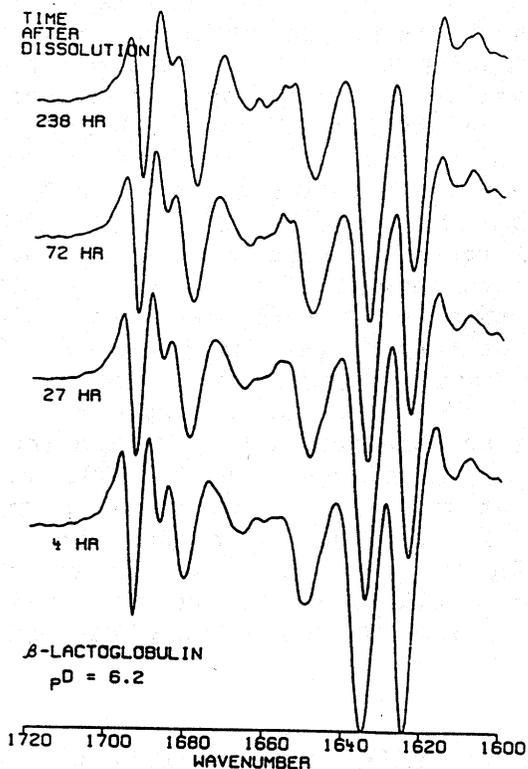


Fig.1. DR2-FTIR spectra of  $\beta\text{LG}$  at pD 6.2.

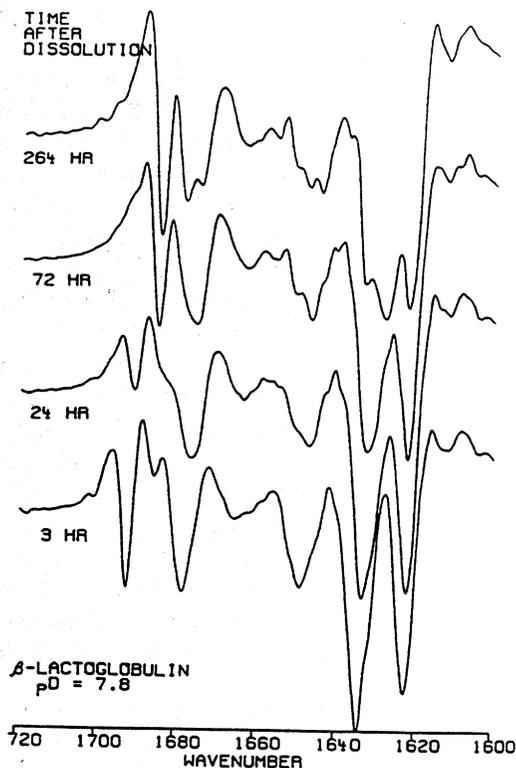


Fig.2. DR2-FTIR spectra of  $\beta\text{LG}$  at pD 7.8.

(and possibly the 1685 and 1692  $\text{cm}^{-1}$  peaks also) may be due to various turns (12%). [Note: 11 residues assigned in reference [8] both to  $\beta$ -sheets and to turns, were counted here only for the former, so that the total would sum to 100%.]

By contrast, when a 3% or 0.5% (w/v) solution of this whey protein is held for 11 days at pD 7.8 (Fig. 2), a number of significant alterations appear in the spectrum. In particular the sharp band at 1692  $\text{cm}^{-1}$  disappears, along with a very weak feature at 1685  $\text{cm}^{-1}$ . A new sharp peak grows in at 1684  $\text{cm}^{-1}$ , and a very weak peak appears at 1674  $\text{cm}^{-1}$  as a shoulder on the original 1678  $\text{cm}^{-1}$  component. A weak feature arises at 1655  $\text{cm}^{-1}$ . Moreover, the intense band at 1633  $\text{cm}^{-1}$  wanes while a new component waxes at 1628  $\text{cm}^{-1}$ . Yet, despite these obvious changes, the relative proportions of  $\beta$ -strands and  $\alpha$ -helices in the protein backbone at pD 7.8 qualitatively appear to remain more or less unaltered even after more than 11 days at room temperature.

One might be tempted simply to attribute these changes to isotopic frequency shifts expected if the increased alkalinity were to promote H/D exchange of residual "hard-to-exchange" amide N-H groups. Were this the case, the observed variation in frequency should proceed gradually and smoothly, without the sudden, discontinuous decreases found at pD 7.8. In addition, because increased temperature facilitates H/D exchange of the peptide protons, similar changes should occur in the spectra after a pD 6.2 protein solution has been heated for a time at a temperature below that which induces irreversible denaturation. In a separate study where  $\beta$ LG was heated to 60  $^{\circ}\text{C}$  for one hour at pD 6.2, the 1692  $\text{cm}^{-1}$  band does lose about 80% of its original peak intensity in the DR2-FTIR [9]. But one observes little evidence of any other significant changes in the Amide I region of the spectrum. Hence, in this case mild heating apparently causes relatively little, if any, irreversible change in the conformation of the protein. In particular, no new bands appear at 1628, 1674, or 1684  $\text{cm}^{-1}$ , such those as seen at pD 7.8 and room temperature (Fig. 2). So it seems unlikely that these new bands originate from isotopic exchange.

Instead, the observed amide I band changes at pD 7.8 must result from changes in the secondary structure of  $\beta$ LG. At pD 6.2, the protein is dimeric [5,6]. The X-ray study implicates one of the nine  $\beta$ -strands as being directly involved in stabilization of the dimer via the formation of intermolecular hydrogen bonds [8]. Above pD 7.5, after monomerization, the intramolecular hydrogen bonds between this  $\beta$ -strand and its neighbors apparently alter considerably, probably becoming stronger. This in turn could explain the conspicuous loss of intensity for the 1634  $\text{cm}^{-1}$  band and the appearance of a new  $\beta$ -band at 1628  $\text{cm}^{-1}$ , 6  $\text{cm}^{-1}$  below the former, as well as the new shoulder at 1674  $\text{cm}^{-1}$ .

This leaves only the changes between 1695 and 1682  $\text{cm}^{-1}$  to be interpreted. One possibility is that these result from turns which have undergone structural alterations. But another alternative exists. Early studies noted that the Tanford transition in  $\beta$ LG is associated with the ionization of a protected (buried) carboxyl group with an anomalously high  $\text{pK}_a$  [5-6]. Recently, Casal et al. [4] reported the infrared spectrum of the  $\beta$ LG B under ambient conditions as a function of pH from 2

to 13. Although they made no attempt to observe any time-dependent changes associated with the Tanford transition near pH 7.5, they did suggest that the 1693  $\text{cm}^{-1}$  band may not be an amide I component but is due to carboxyl C=O stretching vibrations. If their hypothesis is correct, then the disappearance of this band with time at pD 7.8 results from the partial unfolding of the peptide chain so that the anomalous carboxyl group is exposed and more easily ionized. This assumption further suggests that heating the protein to 60 °C at pD 6.2 [9] also renders this carboxyl group more susceptible to dissociation, but with little noticeable overall change in conformation. A difference spectrum (calculated by subtracting the spectrum collected after 3 hr from the one recorded at 264 hr elapsed time) provides further possible corroboration of this supposition. The difference spectrum shows new bands at 1588 and 1435  $\text{cm}^{-1}$  in addition to the changes already noted in the amide I region. In contrast, the analogous difference spectrum at pD 6.2 shows no evidence of new bands or other changes between 1600 and 1300  $\text{cm}^{-1}$ . Thus, these two new bands at pD 7.8 are presumably the antisymmetric and symmetric stretches of the newly ionized carboxylate moiety.

#### CONCLUSION

The invariance of the DR2-FTIR spectra with time suggests that  $\beta$ LG has a stable structure at pD 6.2. By contrast, changes in the spectra of the protein at pD 7.8 cannot simply be attributed to isotopic shifts, but point to a modified secondary structure. In addition, the disappearance of the 1693  $\text{cm}^{-1}$  band along with the manifestation of weak, new bands at 1588 and 1435  $\text{cm}^{-1}$  can be rationalized as resulting from the ionization of an anomalous carboxyl that becomes exposed during the structural transition.

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