

## Investigation of the Carboxyl Ionization of $\beta$ -Lactoglobulin by Differential Infrared Spectroscopy

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### INTRODUCTION

It has been shown that the ionization of carboxyl groups of proteins in aqueous solution can be observed by infrared spectroscopy if deuterium oxide is used as a solvent (1). The substitution of  $D_2O$  for  $H_2O$  permits examination of absorption spectra in the region where characteristic carbonyl, carboxyl, and carboxylate ion bands occur (2). The un-ionized carboxyl groups of  $\beta$ -lactoglobulin absorb around  $1709\text{ cm.}^{-1}$  (1). The band appears as a shoulder on a much stronger band which centers around  $1645\text{ cm.}^{-1}$  and is associated with the  $C=O$  groups of the peptide links.

Recently Tanford, Bunville, and Nozaki have concluded from titration experiments (3) that  $\beta$ -lactoglobulin contains two carboxyl groups which have an apparent  $pK$  value ( $\sim 7.5$ ) much higher than the normal ones. From a combination of titration, optical rotation, and sedimentation data, they have concluded that these carboxyl groups are buried within the molecule and become available to ionization only after a molecular rearrangement which occurs between pH 7 and 8. If the protein is denatured by exposure to pH 11, the anomalous ionization disappears and these two groups ionize in the normal carboxyl range (3).

The purpose of the present study was to determine whether infrared absorption around  $1710\text{ cm.}^{-1}$  can be used for the detection of such abnormal carboxyl groups.

### EXPERIMENTAL

The  $\beta$ -lactoglobulin used in this study was a sample prepared from pooled milk by the method of Palmer (4) and kindly given to us by Dr. W. G. Gordon. For the purpose of this study, the slurry of protein crystals in distilled water was lyophilized, suspended in  $D_2O$  for 3 days and again lyophilized. A 94.5 g./l. solution of the deuterium-substituted protein was prepared using a 0.10 ionic strength KCl solution in

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heavy water as solvent. Solutions of the proper  $pD$ 's were obtained by adding a calculated amount of DCl or NaOD in  $D_2O$ . The solutions of DCl and NaOD were standardized by conventional methods. Because of scarcity of material, the final titration could be carried out only in a semiquantitative way. The calculated and measured  $pD$  values of the final solutions agreed within  $\frac{1}{10}$  of a  $pD$  unit, indicating that deuteration does not result in marked deviation from normal ionization behavior. The final ionic strengths varied between 0.13 and 0.19. The final protein concentration in all cases was 71.0 g./l. In all, samples were prepared at the  $pD$ 's of 9.7, 7.5, 6.8, 5.3, and 3.2.

The denatured samples were prepared by first adding the amount of NaOD calculated to adjust the  $pD$  to approximately 11 and, after the desired period of time, adding enough DCl to bring the  $pD$  back to the neutral region.

A sealed sample cell of conventional design, equipped with  $CaF_2$  windows, a polyethylene spacer, and Teflon sample-intake and outlet connections was constructed. The spacer was sealed to the windows by heating the assembled cell gradually to  $140^\circ C$ . and subsequent cooling back to room temperature. Very slow heating and cooling is necessary if cracking of the  $CaF_2$  windows is to be avoided. The thickness of the cell was about 0.08 mm. A Beckman model IR-3 double monochromator instrument<sup>2</sup> equipped with NaCl prisms was used for making the measurements. The infrared spectrum of the  $pD$  9.7 sample was recorded on tape from 1600 to  $1800\text{ cm}^{-1}$ , and the transmittance of all other samples in the same region was measured with the spectrum of the  $pD$  9.7 sample as reference. The instrumental settings were as follows: gain  $10 \times 1000$  for making the recording,  $10 \times 700$  for measuring differential spectra; speed 32 sec./slit width; time constant 16 sec.; slit width at  $1710\text{ cm}^{-1}$ , 0.173 mm.

The IR-3 instrument compensates for absorption of the reference sample by automatically varying the slit widths according to the prerecorded tape. The method has the advantage of high sensitivity (resulting from constant reference energy) at the expense of frequent changes of the spectral slit width which are not easy to evaluate and can have a distorting effect on band shapes.

Small  $H_2O$  impurities in the deuterated solvent could cause complications because of the H—O—H bending frequency which occurs in the  $1640\text{ cm}^{-1}$  region. Fortunately,  $H_2O$  exchanges very rapidly with  $D_2O$  according to the equation  $H_2O + D_2O \rightleftharpoons 2\text{HOD}$ . The HOD bending mode absorbs at considerably lower frequencies, and, if the amount of initial  $H_2O$  is small, the equilibrium lies very far on the favorable side.

## RESULTS AND DISCUSSION

Figure 1A gives the transmittance of the  $pD$  9.7 sample and the  $pD$  3.2 sample from 1600 to  $1800\text{ cm}^{-1}$  as measured against vacuum. The band caused by the un-ionized carboxyl groups of the latter sample is visible as a shoulder on the strong peptide band. Figure 1B represents the differential spectra of the native samples at various  $pD$  values as measured against the recorded spectrum of the  $pD$  9.7 sample. In this figure, transmittance values above 100% indicate, of course, that at a particular wavelength the sample absorbs less than the reference. The following general observations can be made:

<sup>2</sup> Mention of this company does not constitute an endorsement of its product to the possible detriment of other companies not mentioned.

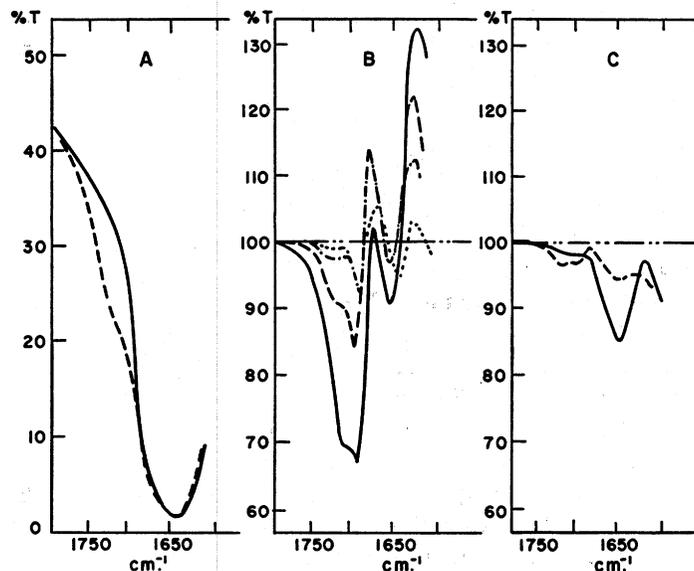


FIG. 1. Infrared absorption spectra of  $\beta$ -lactoglobulin.

A. Transmittance of pD 9.7 sample (solid line) and pD 3.2 sample (dotted line) with vacuum as reference.

B. Differential spectra measured with pD 9.7 sample as reference: ..... pD 7.5; - - - - pD 6.8; — — pD 5.3; — pD 3.2; — · — · pD 9.7 (reference).

C. Differential spectra of samples exposed to pD 11: — — exposed for 2 min.; — exposed for 30 min.

1. The roughly M-shaped curves between 1600 and 1690  $\text{cm}^{-1}$  indicate that the shape of the strong peptide band changes with pD. It appears that at higher pD values the band is narrower and probably has a shoulder around 1690  $\text{cm}^{-1}$ . In solid proteins the position and shape of this band depend on the configuration of the protein chains (5), or, probably more exactly, on the nature of the hydrogen bonds which are possible in a given configuration (6). In  $\text{D}_2\text{O}$  solution the nature of the hydrogen bonds involving peptide groups is certainly different from those in the solid state, and changes in the band shape caused by varying the pD of the solvent are not surprising. The changes are nevertheless very small and can be detected only with great difficulty on nondifferential curves.

2. A shoulder is observed around 1710  $\text{cm}^{-1}$  which decreases with increasing pD, in agreement with the results of previous workers (1).

3. This shoulder is still quite significant at pD 6.8, or even at 7.5, where practically all normal carboxyl groups should be ionized.

4. The position of the shoulder appears to shift from 1707  $\text{cm}^{-1}$  at pD

3.2 to about 1716  $\text{cm}^{-1}$  at  $pD$  7.5, indicating either that the residual groups are in a different intramolecular environment from the normal groups, or that the change in external environment is sufficient to cause the band to shift (or both).

The differential absorbances were first evaluated at the approximate centers of the shoulders, i.e., at 1707  $\text{cm}^{-1}$  for the  $pD$  3.18 sample, at 1713  $\text{cm}^{-1}$  for the  $pD$  5.28 sample, and at 1716  $\text{cm}^{-1}$  for the other samples. To evaluate the effect produced by deviation from a constant frequency, the absorbances of all samples were also evaluated at 1707  $\text{cm}^{-1}$  (center of the shoulder of the  $pD$  3.18 sample). The results agreed within measurement accuracy with the first set of data. This can be qualitatively verified by examining Figs. 1B and 1C, which show that a small frequency shift from the estimated centers to 1707  $\text{cm}^{-1}$  has only a very small influence on transmittance values.

If it is assumed that the C=O absorbance per COOD group is approximately constant, even though the center of the band changes slightly with  $pD$ , it becomes possible to compare the peak intensity of the band as a function of  $pD$  with the number of un-ionized groups present at these  $pD$ 's.

The fraction of ionized groups,  $\alpha$ , at any given pH is expressed by (7):

$$\log \frac{\alpha}{1-\alpha} = \text{pH} - \text{p}K + 0.868\omega\bar{Z} \quad (1)$$

$$\omega = \frac{e^2}{2DkT} \left( \frac{1}{b} - \frac{\kappa}{1+\kappa a} \right)$$

where  $\bar{Z}$  is the average net charge on the protein molecule,  $e$  is the protonic charge,  $D$  is the dielectric constant of the medium,  $k$  is Boltzmann's constant,  $T$  is the thermodynamic temperature,  $b$  is the radius of the protein molecule,  $a$  is the distance of closest approach of the center of a small ion to the center of the protein molecule, and  $\kappa$  is the reciprocal thickness of the ionic atmosphere.

According to Tanford *et al.* (3), a molecule of  $\beta$ -lactoglobulin (molecular weight = 35,000) has 48.5 side-chain carboxyls with an intrinsic  $pK$  of 4.7, two terminal carboxyls with an intrinsic  $pK$  of 3.75, and two abnormal carboxyls with an apparent  $pK$  of 7.5. With the help of these data and the above equation, setting  $b = 2.5 \times 10^{-7}$  cm., the number of nonionized groups was calculated for the  $pD$ 's studied by infrared absorption. These results are compared in Table I and in Fig. 2 with the number of such groups deduced from the absorbance at about 1710  $\text{cm}^{-1}$ . This was done with the use of a proportionality constant, obtained by setting the observed absorbance value of 0.15, corresponding to 39.8 groups ( $pD = 3.2$ ), as a standard. As can be seen, a good agreement is obtained between

TABLE I  
*Carboxyl Ionization of  $\beta$ -Lactoglobulin*

pH (pD)	Normal	COOH calculated by Eq. (1) Anomalous	Total	COOD observed
3.18	37.8	2.0	39.8	39.8 <sup>a</sup>
5.28	12.3	2.0	14.3	13.3
6.82	1.1	1.65	2.75	2.5
7.53	0.3	0.95	1.25	1.5
9.70	0	0	0	0 <sup>a</sup>
6.51 <sup>b</sup>	1.8	—	—	3.3
6.81 <sup>c</sup>	1.1	—	—	1.5

<sup>a</sup> Taken as standard.

<sup>b</sup> Exposed 2 min. to pD 11.

<sup>c</sup> Exposed 30 min. to pD 11.

the calculated and experimental values, especially if it is considered that the error in measuring the absorbance corresponds to  $\pm 0.5$  group. These observations can be considered as confirmation of the results of Tanford *et al.* (3) that  $\beta$ -lactoglobulin indeed does have two carboxyl groups which become ionized only in the pH range above 6.5.

The differential spectra of the two samples exposed to pD 11 and brought back to the neutral zone, as measured against the pD 9.7 sample, are shown in Fig. 1C. The sample exposed to pD 11 for 2 min. and readjusted to pD 6.5 shows a 1710  $\text{cm}^{-1}$  region band slightly greater than the native pD 6.8 sample. The band of the sample exposed for 30 min. to pD 11 and readjusted to pD 6.8 is considerably weaker and has an absorbance corresponding to 1.5 un-ionized carboxyls. Assuming no great changes in  $\omega$  as a result of denaturation, one can calculate that there should be 1.1 un-ionized normal carboxyls at pD 6.81. This would leave 0.4 un-ionized anomalous groups after 30 min. of denaturation as compared to 1.65 such groups in the native protein, indicating that denaturation deprives the anomalous groups of their low ionization constant, in agreement with Tanford and co-workers. The 1600–1700  $\text{cm}^{-1}$  region is likewise changed by the alkaline treatment, indicating that the peptide C=O groups are in a different environment from the untreated samples. The sample that had been denatured for 2 min. has an absorbance corresponding to 3.3 un-ionized carboxyls at pD 6.51. At this pD, there should be 1.8 normal groups, leaving 1.5 to be accounted for. The fact that in the native protein one can calculate the presence of 1.8 anomalous un-ionized carboxyl groups at that pD indicates that the short treatment has resulted only in an incipient denaturation, which has not altered irreversibly the conformation that gives rise to the anomalous ionization. Indeed, this sample remained completely

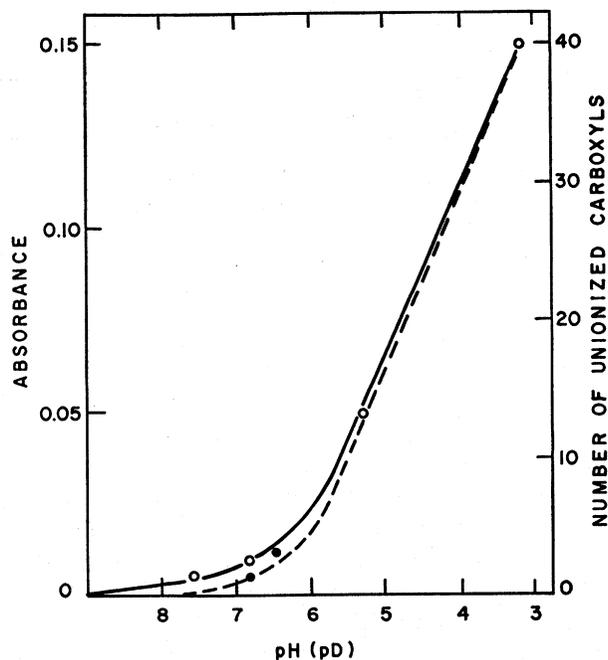


FIG. 2. Absorbance and degree of ionization of  $\beta$ -lactoglobulin carboxyls. Open circles: native protein; filled circles: protein exposed to pD 11. Solid line calculated by Eq. (1) assuming two abnormal carboxyls. Dashed line calculated by Eq. (1) assuming that all carboxyls are normal.

in solution after neutralization, while the half-hour denatured material coagulated on adjustment to pD 6.81.

Although deuteration might have influenced the ionization to some degree, the effect is probably not large enough to have appreciably changed the nature of the curve in Fig. 2 (cf. *Experimental* section). Furthermore, the relative number of un-ionized groups at pD 6.82 and pD 7.53 (as compared with pD 3.18) retains its significance in any case, as do the observations regarding denaturation.

The above-described results would seem to confirm fully the observations of Tanford, Bunville, and Nozaki (3) that  $\beta$ -lactoglobulin has two carboxyl groups which become ionized only in the pH region above 6.5 and that denaturation of the protein by exposure to high pH results in a molecular rearrangement which eliminates these groups. Furthermore, it is possible to conclude that the method of differential infrared absorption can be used to detect abnormal carboxyl groups, even if these are present only in very small amounts.

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#### SUMMARY

A differential infrared study has been carried out as a function of pH on  $\beta$ -lactoglobulin in the region of carboxyl absorption.

The observations of Tanford and co-workers that this protein contains two carboxyl groups that remain un-ionized up to pH 6.5 has been confirmed.

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