Sklenár, V., Miyashiro, H., Zon, G., Miles, H. T., & Bax, A. (1986) *FEBS Lett. 208*, 94–98.
Van De Ven, F. J. M., & Hilbers, C. W. (1988) *Eur. J. Biochem. 178*, 1–38.
Wagner, G., & Wüthrich, K. (1979) *J. Magn. Reson. 33*, 675–680.
Weiner, P. K., & Kollman, P. A. (1981) *J. Comput. Chem. 2*, 287–303.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
Yanagi, K., Prive, G. G., & Dickerson, R. E. (1991) *J. Mol. Biol. 217*, 201–214.

Effect of Metal Ion Binding on the Secondary Structure of Bovine α-Lactalbumin As Examined by Infrared Spectroscopy

Steven J. Prestrelski,*,‡ D. Michael Byler,‡,§ and Marvin P. Thompson‡,∥

Eastern Regional Research Center, U.S. Department of Agriculture, 600 East Mermaid Lane, Philadelphia, Pennsylvania 19118, and Department of Chemistry and Physical Science, Philadelphia College of Textiles and Science, Schoolhouse Lane and Henry Avenue, Philadelphia, Pennsylvania 19144

Received February 22, 1991; Revised Manuscript Received June 6, 1991

ABSTRACT: We have examined the influence of monovalent and divalent cations on the secondary structure of bovine α-lactalbumin at neutral pH using Fourier-transform infrared spectroscopy. Our present studies are based on previously reported amide I′ component band assignments for this protein [Prestrelski, S. J., Byler, D. M., & Thompson, M. P. (1991) *Int. J. Pept. Protein Res. 37*, 508–512]. The results indicate that upon dissolution, α-lactalbumin undergoes a small, but significant, time-dependent conformational change, regardless of the ions present. Additionally, these studies provide the first quantitative measure of the well-known secondary structural change which accompanies calcium binding. Results indicate that removal of Ca^{2+} from holo α-lactalbumin results in local unfolding of the Ca^{2+}-binding loop; the spectra indicate that approximately 16% of the backbone chain changes from a rigid coordination complex to an unordered loop. We have also examined the effects of binding of several other metal ions. Our studies have revealed that binding of Mn^{2+} to apo α-lactalbumin (Ca^{2+}-free), while inducing a small, but significant, conformational change, does not cause the α-lactalbumin backbone conformation to change to that of the holo (Ca^{2+}-bound) form as characterized by infrared spectroscopy. Similar changes to those induced by Mn^{2+} are observed upon binding of Na^+ to apo α-lactalbumin, and furthermore, even at very high concentrations (0.2 M), Na^+ does not stabilize a structure similar to the holo form. Binding of Zn^{2+} to the apo form of α-lactalbumin does not result in significant backbone conformational changes, suggesting a rigid Zn^{2+}-binding site. Further, as characterized by infrared spectroscopy, binding of Zn^{2+} to holo α-lactalbumin does not induce a reversion to an apolike conformer. These results suggest that current models of the metal-binding properties of α-lactalbumin need to be carefully reexamined in light of these findings.

α-Lactalbumin is a protein present in the whey portion of the milk from most species of mammals. It functions as a modifier protein in lactose biosynthesis where it directs the specificity of the enzyme galactosyltransferase (Brodbeck et al., 1967). In addition to its physiological roles, α-lactalbumin has been demonstrated to exhibit potent antitumor activity in human mammary carcinoma cell lines (Bano et al., 1985). The structural integrity of α-lactalbumin may have important implications in these and other biological processes. Previous investigations have demonstrated a strong influence of monovalent and divalent cations on the conformation, stability, and activity of α-lactalbumin. Several studies have demonstrated that this protein has a strong Ca^{2+}-binding site whose binding constant is on the order of 10^{-8}–10^{-9} M (Berliner & Johnson, 1988). Intrinsic fluorescence and aromatic circular dichroism (CD)[1] studies have suggested that binding of Ca^{2+} to this site results in a marked conformational change (Permyakov et al., 1981a; Segawa & Sugai, 1983). Further, Ca^{2+} has been demonstrated to enhance the thermal stability of α-lactalbumin (Kuwajima et al., 1986). Recently, the atomic structure of baboon α-lactalbumin has been determined at a resolution of 1.7 Å by X-ray crystallography (Acharya et al., 1989). This study revealed an extremely well-formed, high-affinity Ca^{2+}-binding site, different from the previously observed EF-hand motif (Stuart et al., 1986).

Other investigations of α-lactalbumin have demonstrated binding of other cations to the Ca^{2+} site, including Mg^{2+}, Mn^{2+}, Na^+, and K^+ (Permyakov et al., 1981b, 1985; Murakami & Berliner, 1983; Segawa & Sugai, 1983). A second, distinct cation-binding site has been implicated for α-lactalbumin which has a specificity for Zn^{2+} (Murakami & Berliner, 1983). This study has also suggested that binding to the Ca^{2+} and Zn^{2+} sites is mutually exclusive. That is, binding of Zn^{2+} to holo α-lactalbumin causes displacement of bound Ca^{2+} and

* To whom correspondence should be addressed at Amgen Inc., Amgen Center, 1840 Dehavilland Dr., Thousand Oaks, CA 91320.
‡ U.S. Department of Agriculture.
§ Philadelphia College of Textiles and Science.
∥ Present address: 1432 Manor Lane, Bluebell, PA 19422.

[1] Abbreviations: CD, circular dichroism; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; IR, infrared; FTIR, Fourier-transform infrared spectroscopy; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

0006-2960/91/0430-8797$02.50/0

a subsequent reversion of the conformation to the apo form. A conformational change was proposed as a mechanism for this Zn^{2+}-induced displacement. However, later studies have concluded that Ca^{2+} and Zn^{2+} can bind to α-lactalbumin simultaneously, although its fluorescence characteristics are similar to that of apo α-lactalbumin (Musci & Berliner, 1985a,b). Thus, it is apparent that more detailed knowledge of the effects of metal ions on the conformational states of α-lactalbumin is essential to understanding the structure–function relationships of this complex molecule.

Infrared spectroscopy, with the advent of Fourier-transform spectrometers and resolution-enhancement (band-narrowing) algorithms, has become a widely used method for the examination of protein secondary structure in solution [see Surewicz and Mantsch (1988) for a review]. Most infrared studies of protein conformation focus on absorptions in the amide I (designated amide I′ in deuterated peptides and proteins) region (1700–1620 cm^{-1}) which arise primarily from stretching vibrations of the backbone C=O groups (Miyazawa & Blout, 1961). The frequency of these vibrations has been shown to be sensitive to the molecular geometry and hydrogen bonding characteristics of the peptide backbone (Krimm & Bandekar, 1986), and furthermore, for a wide variety of proteins, specific secondary structures (helix, strand, turns, etc.) give rise to discrete bands that consistently appear in just a narrow portion of the amide I′ region (Byler & Susi, 1986). However, for most polypeptides and proteins, the separation between these component bands is frequently less than their inherent bandwidths. Thus, one typically observes only a single, undifferentiated composite amide I band which both is quite broad and often appears featureless. Because these bandwidths are inherently large, increasing the instrumental resolution is to no avail. Thus, mathematical methods termed resolution enhancement or band-narrowing techniques, including derivative spectroscopy (Susi & Byler, 1983) and Fourier self-deconvolution (Byler & Susi, 1986), have been applied to visualize the individual amide I components in protein spectra. The combination of resolution-enhancement methods with the judicious application of curve-fitting techniques frequently reveals a wealth of information concerning the secondary structure of proteins (Byler & Susi, 1986; Lee & Chapman, 1986; Surewicz & Mantsch, 1988). Similar results concerning secondary structure have been obtained from the amide I region of infrared spectra by several alternative experimental and data analysis methods (Dong et al., 1990; Dousseau & Pezolet, 1990; Goormaghtigh et al., 1990; Lee et al., 1990).

Recent results from our lab have demonstrated the high degree of sensitivity of resolution-enhanced FTIR spectroscopy to subtle changes in protein conformation (Byler & Purcell, 1989; Prestrelski et al., 1991b). In another study, we have reported a comparative spectroscopic study of α-lactalbumin and the homologous protein lysozyme in which infrared results were correlated with information from the known crystallographic structures of these two proteins (Prestrelski et al., 1991a). This study resulted in assignment of the secondary structures in α-lactalbumin to its amide I′ infrared component bands, with particular insight into the IR characteristics of 3_{10}-helices. In the present paper, we report the results of an infrared spectroscopic examination of the influence of monovalent and divalent cation binding on the secondary structure of α-lactalbumin.

Experimental Procedures

Materials. α-Lactalbumin [type I (~1.1–1.3 mol of Ca^{2+}/mol of protein) and type III (calcium-depleted), from bovine milk] was purchased from Sigma Chemical Co. and used without further purification. The purity of α-lactalbumin was verified electrophoretically by using SDS–PAGE and nondenaturing PAGE. The type III α-lactalbumin from Sigma which was used in these studies was determined to contain 0.08 mol of Ca^{2+}/mol of protein by atomic absorption spectroscopy. D_2O was purchased from Cambridge Isotopes Inc., 99.90% isotopic purity. All other chemicals used were reagent grade.

Sample Preparation. For infrared spectroscopy, proteins were prepared as 3.5% (w/v) solutions in 20 mM imidazole buffers (pD = 6.9) prepared with D_2O. The pD of D_2O solutions was determined by adding 0.4 unit to the pH measured with a glass electrode (Covington et al., 1968). The pure apo form of α-lactalbumin was obtained by addition of small amounts of EGTA (1 mM) to solutions of type III α-lactalbumin as described in Murakami et al. (1982). In studies of Zn^{2+} binding to α-lactalbumin, $ZnCl_2$ was added to protein solutions from a dilute stock solution in several small portions to avoid precipitation of the protein. A protein concentration slightly higher than the desired concentration was prepared initially. Small amounts of precipitated proteins were removed by centrifuging the samples for 15 min at 4 °C. The concentration of the protein after centrifuging was then diluted with deuterated buffer to approximately 3.5% as estimated by comparing the amide I′ intensity to that of other α-lactalbumin solutions.

Protein and buffer solutions were placed in 75-μm pathlength IR cells with CaF_2 windows and Teflon spacers. A previous report of an infrared examination of Ca^{2+}-binding proteins carefully checked for the possible contamination from CaF_2 windows (Trewhella et al., 1989). This study concluded that no calcium resulting from the window material was detectable in the sample buffer using atomic absorption spectroscopy. Additionally, EGTA was added to the buffer solutions when it was imperative that no Ca^{2+} be present in the solutions.

Spectroscopy. Infrared 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, liquid nitrogen cooled mercury/cadmium telluride detector. All spectra were recorded at a resolution of 2 cm^{-1} by collecting 4096 co-added, double-sided interferograms (0.44 s/scan) which were Fourier-transformed after application of a Happ–Genzel apodization function. (In time course studies, the first spectrum was collected by averaging only 512 scans.) The spectrometer and sample chamber were purged continuously with dry nitrogen gas. Spectral contributions from residual H_2O vapor in the light path and from buffer components were subtracted by using programs provided with the Nicolet FTIR software, version 4.3. Factors for water vapor subtraction were determined by subtracting a second-derivative spectrum of water vapor from the second-derivative spectrum of the sample. The subtraction factor was varied until the absorption-free region above 1700 cm^{-1} was featureless.

Analysis of Spectra. The analysis of protein infrared spectra was the same as that described previously (Prestrelski et al., 1991b). Briefly, the second-derivative spectra were calculated analytically as described by Susi and Byler (1983), except that the first-derivative function was applied twice. Thus, each of the second-derivative data points is calculated over five data points rather than three. Fourier self-deconvolutions were also carried out by using the Nicolet software which is based on the method of Kauppinen et al. (1981). For these spectra of α-lactalbumin, deconvolution parameters of 18 cm^{-1} and 2.8

Table I: Frequencies (in cm^{-1}) and Relative Intensities of the Amide I′ Component Bands of α-Lactalbumin in the Presence of Calcium at Various Times after Dissolution[a]

15 min		1 h		8 h		24 h		48 h		72 h		96 h	
ν	A	ν	A	ν	A	ν	A	ν	A	ν	A	ν	A
1633	0.18	1633	0.19	1633	0.20	1630	0.17	1630	0.14	1629	0.13	1629	0.13
1642	0.21	1642	0.21	1641	0.21	1640	0.26	1640	0.27	1639	0.29	1639	0.30
1651	0.25	1651	0.25	1651	0.25	1651	0.23	1651	0.32	1651	0.22	1651	0.23
1660	0.18	1660	0.19	1659	0.17	1659	0.18	1659	0.18	1659	0.14	1659	0.16
1669	0.11	1669	0.11	1669	0.12	1668	0.08	1668	0.07	1668	0.08	1668	0.08
1680	0.05	1680	0.04	1680	0.04	1677	0.07	1677	0.07	1677	0.07	1677	0.07
		1689	0.01	1688	0.01	1687	0.02	1687	0.02	1686	0.02	1686	0.02
1692	0.02	1695	0.01	1696	0.01								

[a] The times refer to the midpoint of the data collection period.

Table II: Frequencies (in cm^{-1}) and Relative Intensities of the Amide I′ Component Bands of α-Lactalbumin in the Absence of Calcium at Various Times after Dissolution[a]

15 min		1 h		8 h		24 h		48 h		72 h		96 h	
ν	A	ν	A	ν	A	ν	A	ν	A	ν	A	ν	A
1630	0.13	1629	0.14	1629	0.14	1629	0.14	1629	0.13	1629	0.12	1629	0.12
1638	0.20	1638	0.20	1638	0.22	1638	0.22	1638	0.23	1638	0.22	1638	0.22
1647	0.25	1646	0.22	1646	0.18	1646	0.16	1646	0.16	1646	0.17	1645	0.16
1653	0.09	1654	0.14	1654	0.20	1654	0.23	1654	0.26	1654	0.24	1654	0.27
1662	0.25	1662	0.20	1664	0.14	1664	0.12	1665	0.10	1664	0.12	1665	0.10
1677	0.08	1677	0.09	1676	0.10	1675	0.09	1675	0.09	1675	0.09	1674	0.09
1683	0.01												
		1685	0.01	1684	0.01	1683	0.03	1683	0.03	1683	0.04	1683	0.04
1693	0.01	1693	0.01	1693	0.01								

[a] The times refer to the midpoint of the data collection period.

for the undeconvoluted band half-width and resolution enhancement factor, k, respectively, were found to be optimal. A Lorentzian line-shape function and a Bessel apodization function were used. Curve fitting was performed with the program ABACUS, an iterative Gauss–Newton nonlinear regression program [see Byler and Susi (1986)]. Deconvoluted spectra were fitted with Gaussian band profiles. Initial band positions were taken directly from the second-derivative spectra, and no additional bands were added unless clearly resolved in the corresponding deconvoluted spectrum. Initial values for the peak heights and widths were estimated from spectra. A nonsloping base line was estimated from the 1700–1800 cm^{-1} region of the spectrum and held constant. For the final fits, the heights, widths, and positions of all bands were varied simultaneously. The relative integrated intensity of each band (i.e., the band area as a fraction of the total amide I′ area) was then calculated from the final fitted band heights and widths.

Results and Discussion

Tables I and II list the peak positions and calculated relative integrated intensities for the deconvoluted spectrum of α-lactalbumin at various times after dissolution in the presence and absence of Ca^{2+}, respectively. The assignments of these component bands are the same as those reported in Prestrelski et al. (1991a) with the exceptions of the bands near 1646 and 1695 cm^{-1}. The former appears only in the absence of Ca^{2+}, and the latter was not observed in the previous study. According to earlier investigations, bands near 1646 cm^{-1} in spectra of proteins in D_2O are associated with irregular conformations, or conformations in which the backbone folding pattern shows no apparent regularity (Byler & Susi, 1986; Surewicz & Mantsch, 1988). A more recent study has demonstrated that disordered conformations, regions of the protein molecule where no significant electron density is observed in X-ray diffraction experiments, also absorb near this frequency and that the two cannot be distinguished with present methods (Prestrelski et al., 1991b). The weak feature at 1695 cm^{-1} remains unassigned as previous reports have raised questions as to the origin of similar bands (Casal et al., 1989; Byler & Purcell, 1989). These studies have provided evidence that, for some proteins, bands near this frequency may not arise from amide I′ vibrations but from an anomalous, unionized side-chain carboxyl group. Thus, with the present information, we cannot assign this band with confidence.

Several of the component bands listed in Tables I and II reveal significant shifts in the positions and intensities of several of the amide I′ component bands as a function of time. These changes are also apparent in the second-derivative spectra shown in Figure 1. Some of the amide I′ components of proteins in D_2O solutions often undergo small frequency shifts (1–2 cm^{-1}) with time shortly after dissolution because of changes in the vibrational frequencies due to exchange of labile backbone hydrogen atoms for deuterium atoms (Susi, 1969; Haris et al., 1986; Olinger et al., 1986; Byler & Purcell, 1989). However, the shifts observed here cannot be explained by deuteration effects alone. Shifts in amide I vibrational frequencies due to H–D exchange are always to lower frequency. However, a band near 1683 cm^{-1}, present in the initial spectra of apo α-lactalbumin, disappears, and a new, higher frequency band becomes apparent near 1685 cm^{-1}. Similarly, in later spectra of the holo α-lactalbumin, a band becomes apparent near 1689 cm^{-1} which is not observed in the initial spectrum. Further, in the spectra of both the apo and holo forms, a peak near 1695 cm^{-1}, which is present in the spectra taken at earlier times, disappears within 24 h. Although, as stated in the previous paragraph, the origin of this rather weak band is unclear, in both samples its abrupt disappearance is indicative of a small structural change. In addition, several alterations in the relative intensities of the amide I′ components are observed with time. Thus, the spectroscopic results indicate that upon dissolution, α-lactalbumin undergoes a small but significant conformational transition. The magnitude of this change cannot be estimated due to the confounding effects of the observed deuteration shifts. The spectra indicate that this transition is essentially complete within 24 h. Other, small

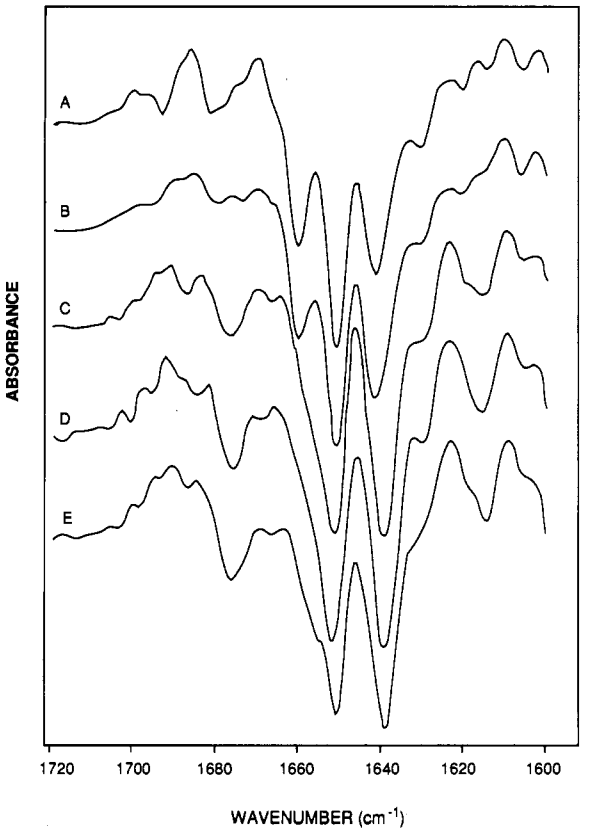

FIGURE 1: Second-derivative spectra of Ca^{2+}–α-lactalbumin at various times after dissolution in D_2O buffer: (A) 15 min; (B) 1 h; (C) 8 h; (D) 36 h; (E) 72 h.

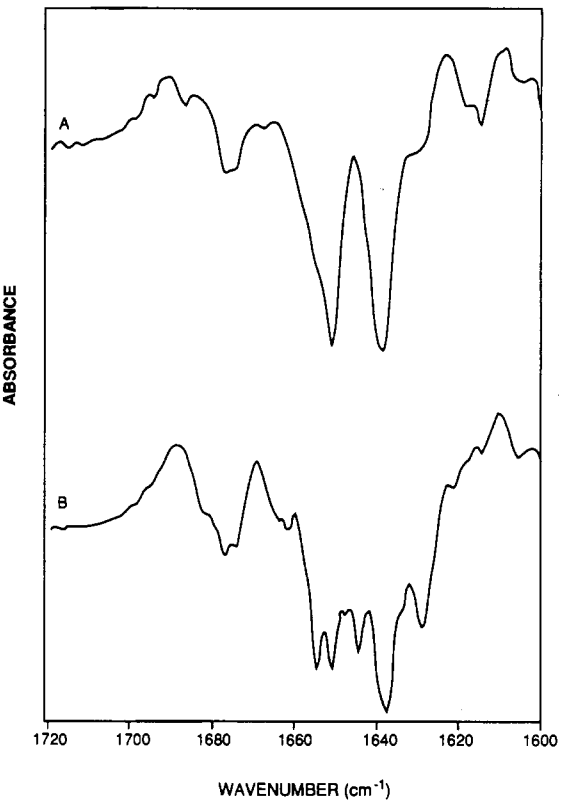

FIGURE 2: Second-derivative spectra of α-lactalbumin. (A) pD = 6.9, 20 mM $CaCl_2$; (B) pD = 6.9, 1 mM EGTA. (Elapsed time is 48 h after dissolution.)

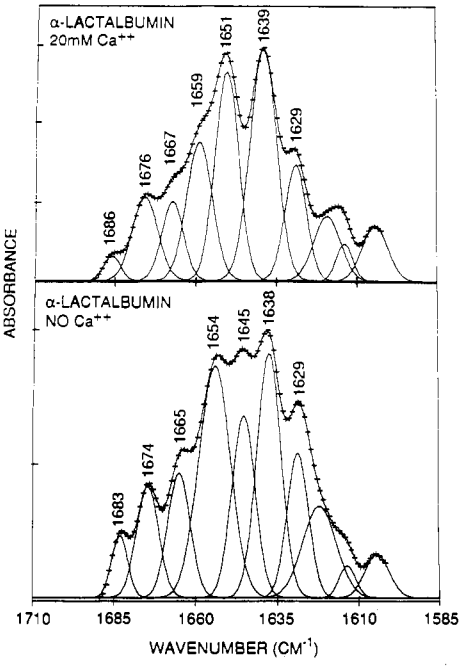

FIGURE 3: Spectra of α-lactalbumin after Fourier self-deconvolution (+). Individual Gaussian components and their sum as determined by curve-fitting (—). (Top) pD = 6.9, 20 mM $CaCl_2$; (bottom) pD = 6.9, 1 mM EGTA. Note: several bands arising from side-chain vibrations (Chirgadze et al., 1975) are fitted below 1620 cm^{-1} which are not amide I′ components. These are added to the analysis to eliminate the approximation otherwise incurred by addition of a sloping base-line parameter.

spectral changes which are apparent after this point can be explained as being due to further deuteration. Although the exact nature of the observed conformational transition cannot be determined with the present information, one possibility is that the protein takes an extended time period to completely dissolve. It appears that after this initial transition, the conformation of α-lactalbumin is stable for several days at room temperature. This small, slow conformational change is similar to that reported for another milk protein, β-lactoglobulin (Byler & Purcell, 1989).

Binding of Calcium. Tables I and II also indicate a dependence of the amide I peak positions and intensities on the presence or absence of Ca^{2+}. This is also evident in Figures 2 and 3 which show, respectively, the second-derivative spectra and curve-fitted, deconvoluted spectra of the Ca^{2+}-bound (holo) and the Ca^{2+}-free (apo) forms of α-lactalbumin. The spectra considered here are those taken well after the conformational transition has apparently completed (~48 h after dissolution). The most apparent difference between the two spectra is the presence of a strong band at 1646 cm^{-1} in the spectrum of apo α-lactalbumin, not present in the spectrum of the holo form, which accounts for 0.16 of the relative integrated intensity of the amide I′ band in the former case. Additional spectroscopic differences are observed between the spectra of the two forms. The band near 1639 cm^{-1} decreases in relative integrated intensity by 0.06 in the absence of bound calcium. Further, while two distinct bands are observed near 1659 and 1651 cm^{-1} in the deconvoluted spectrum of holo α-lactalbumin, only one is seen near 1654 cm^{-1} in the apo form. Two bands are observed near 1651 and 1655 cm^{-1} in the second-derivative spectrum of apo α-lactalbumin (Figure 2), but apparently this frequency difference (4 cm^{-1}) relative to the widths of the two bands is too small for them to be separated by deconvolution in the spectrum of the apo form. Thus, the deconvoluted spectra give only a single α-helical peak for apo α-lactalbumin. The two features in the spectrum of apo α-lactalbumin are therefore fitted as a single peak.

Overall, the loss in the relative integrated intensity from the components attributed to α-helices is 0.16 in the absence of Ca^{2+}. In addition to these differences, a peak is present at 1686 cm^{-1} in the spectrum of the holo form, which appears at 1683 cm^{-1} in the spectrum of the apo form. This suggests alterations in the region of a reverse turn (Prestrelski et al., 1991a). These results are generally in agreement with previous intrinsic fluorescence (Permyakov et al., 1981a) and aromatic CD studies (Segawa & Sugai, 1983) which indicate a distinct change in spectral properties upon Ca^{2+} binding. However, as has become apparent in our infrared studies of Mn^{2+} and Na^+ binding to α-lactalbumin, a change in conformation may not be entirely responsible for the observed differences in the fluorescence and aromatic CD properties.

As stated above, bands near 1646 cm^{-1} are associated with disordered or irregular structures. Amide I′ components in the region of 1650–1659 cm^{-1} arise from α-helices (Byler & Susi, 1986; Surewicz & Mantsch, 1988) while component absorptions near 1638 cm^{-1} have recently been attributed to 3_{10}-helices (Holloway & Mantsch, 1989; Prestrelski et al., 1991a). Thus, the changes observed in the relative integrated intensities of these bands (after 48 h; see Tables I and II) imply an increase in disordered structures and a concomitant decrease in α- and 3_{10}-helical structures upon removal of calcium from α-lactalbumin. On the basis of the recently determined crystal structure of holo α-lactalbumin (Acharya et al., 1989), this information strongly suggests local unfolding of the Ca^{2+}-binding loop in the absence of bound Ca^{2+}. The structure of this loop consists of a 3_{10}-helix leading into an α-helix with a short turn region separating the two helices (Acharya et al., 1989). Several of the amino acid residues of these helices are directly involved in the coordination of the bound Ca^{2+} ion. Indeed, four of the five protein residues which contribute to the coordination of the calcium ion are part of these two helices. In addition, the residues which comprise the Ca^{2+}-binding loop, which is closed off by a disulfide bridge, account for 16% of the total residues. This is equal to the relative integrated intensity of the 1646 cm^{-1} band in the apo form of α-lactalbumin. Thus, results from these studies strongly suggest that removal of bound Ca^{2+} from α-lactalbumin causes a transition in this part of the peptide chain from a rigid, well-defined coordination complex, as observed crystallographically, to an essentially orderless loop. However, while this is the simplest interpretation of these results, other, more complicated interpretations cannot be ruled out.

We have also studied calcium binding at two other Ca^{2+} concentrations, 3 mM (a slight stoichiometric excess) and 200 mM (large excess), in order to examine the effects of possible binding to a second, weaker binding site which has been reported (Kronman et al., 1981; Murakami et al., 1982). (The second, weaker Ca^{2+}-binding site is thought to be the Zn^{2+}-binding site.) The spectra of α-lactalbumin at these concentrations (not shown) are essentially identical with the spectrum collected at 20 mM Ca^{2+} concentration. These data indicate that the conformational transition observed on binding of Ca^{2+} is due only to binding at the strong site, in agreement with previous studies (Kronman et al., 1981). However, these results are in apparent disagreement with a report which demonstrated that the fluorescence characteristics of bis(ANS)–α-lactalbumin at very high Ca^{2+} concentrations are more like the apo conformer (Musci & Berliner, 1985a), suggesting a reversion to an apolike conformer upon binding of Ca^{2+} to the Zn^{2+} site. No changes are observed in the infrared spectra of α-lactalbumin at the higher Ca^{2+} concentrations, indicating that the protein remains in the holo conformation. This apparent discrepancy is probably due to differences in the experimental probes. Whereas IR spectroscopy is sensitive to changes in backbone conformation, the fluorescent probe bis(ANS) is sensitive to hydrophobic surfaces on a protein molecule. Thus, on the basis of the complementary information from these two spectroscopic techniques, it appears that binding of Ca^{2+} to the second site alters the nature of the hydrophobic sites on α-lactalbumin such that it more closely resembles the apo conformer in this respect but that the secondary structure is not appreciably changed.

Additional control studies (results not shown) were carried out which have demonstrated that the presence or absence of EGTA does not produce detectable differences in the amide I′ region of the infrared spectrum of α-lactalbumin. That is, the spectra of type III (Ca^{2+}-depleted) α-lactalbumin in the amide I′ region in the presence or absence of 1 mM EGTA are virtually identical. The spectra of α-lactalbumin with 20 mM $CaCl_2$ in the presence or absence of 1 mM EGTA are also identical with each other. Thus, on the basis of the observed infrared spectra, it appears that neither EGTA nor the Ca^{2+}–EGTA complex causes measurable secondary structure changes to α-lactalbumin under the conditions of this study.

Binding of Manganese. The binding of Mn^{2+} to α-lactalbumin provides an interesting case study. Previous reports, based on intrinsic fluorescence and aromatic circular dichroism studies (Kronman et al., 1981; Murakami et al., 1982; Segawa & Sugai, 1983), have indicated that binding of Mn^{2+} to the Ca^{2+} site results in a conformational change similar to that which is induced by Ca^{2+} binding, although as Murakami et al. (1982) have pointed out, the fluorescence quenching characteristics of Mn^{2+}–α-lactalbumin do not completely approach those of the Ca^{2+} bound form, even at high Mn^{2+} concentrations. This result was interpreted by these investigators as arising from small amounts of apo α-lactalbumin. Additionally, calorimetric studies reveal significant differences in the thermodynamics of Ca^{2+} and Mn^{2+} binding to α-lactalbumin (Desmet & van Cauwelaert, 1988). The molar enthalpy change on binding Mn^{2+} is anomalously low compared to that observed on binding of Ca^{2+}, which may suggest a different mode of binding.

The results of the studies reported here appear to help explain the differences in the spectroscopic and calorimetric results. Table III lists the peak positions and the relative intensities of the amide I′ component peaks for type III (calcium-depleted) α-lactalbumin in the presence of 20 mM Mn^{2+}. These values are generally the same as those of the apo form of α-lactalbumin, although small but significant changes are apparent, particularly for the 1687 cm^{-1} component. Thus, these results indicate that binding of Mn^{2+} does not induce any large backbone conformational change, such as that observed on binding of Ca^{2+}, and that the secondary structure of Mn^{2+}–α-lactalbumin basically resembles the apo (Ca^{2+}-free) form. This may in part explain the differences observed in the fluorescence and calorimetric characteristics upon binding of Mn^{2+} or Ca^{2+}. Much of the observed differences in the fluorescence characteristics of the Ca^{2+}-bound and Ca^{2+}-free forms may be due to the presence of the charged species, per se, and not the observed conformational change. That is, the total difference in fluorescence characteristics is due in small part to a secondary structure change, but in large part to other effects the ion may incur upon interaction with the fluorescent side chains such as electrostatic effects or, possibly, changes in solvent exposure. It is possible, as argued previously (Desmet & van Cauwelaert, 1988), that the smaller

Table III: Peak Positions (cm^{-1}) and Relative Integrated Intensities of the Amide I′ Components of α-Lactalbumin in the Presence of Various Metal Ions[a]

20 mM Mn^{2+}		40 mM Na^+		200 mM Na^+		10 mM Ca^{2+}/2 mM Zn^{2+}		3 mM Zn^{2+}	
ν	A	ν	A	ν	A	ν	A	ν	A
								1622	0.09
1629	0.14	1629	0.12	1629	0.13	1628	0.12	1629	0.13
1638	0.24	1638	0.23	1637	0.23	1638	0.31	1638	0.21
1646	0.17	1646	0.19	1646	0.21			1646	0.11
						1651	0.25		
1654	0.22	1654	0.23	1654	0.18	1658	0.13	1654	0.25
1664	0.13	1664	0.12	1663	0.16	1666	0.08	1665	0.08
1674	0.10	1675	0.10	1676	0.09	1676	0.08	1674	0.09
1687	0.01	1683	0.01	1686	0.01	1685	0.02	1683	0.03

[a] These spectra are those collected at ~48 h after dissolution.

size of the Mn^{2+} ion [r_{Mn} = 67 pm vs r_{Ca} = 100 pm, for a coordination number of 6 (Shannon & Previtt, 1969)] cannot accommodate the near-perfect coordination complex observed crystallographically for the Ca^{2+}-bound species (Stuart et al., 1986) and thus does not result in the backbone conformational transition which is observed upon the binding of Ca^{2+}. This would appear to explain the anomalously large difference in the enthalpy change of binding observed for these two ions (Desmet & van Cauwelaert, 1988). Basing their interpretation on previously reported fluorescence and aromatic CD experiments, Desmet and van Cauwelaert (1988) interpreted the difference in binding enthalpy as a reduced energy of electrostatic stabilization. However, these infrared results would argue that the smaller enthalpy change observed upon binding of Mn^{2+} is due, at least in part, to the absence of a conformational change. This contrasts with the large conformational change that is observed upon binding of Ca^{2+} to α-lactalbumin.

Binding of Sodium. Previous reports have demonstrated that the monovalent ions Na^+ and K^+ bind to α-lactalbumin, but with a much lower affinity than the divalent cations (Hiroaka & Sugai, 1985; Desmet et al., 1987). These studies, which used aromatic CD spectroscopy, showed that at high concentrations of Na^+ or K^+, the spectrum of α-lactalbumin was shifted back to that of the holo (Ca^{2+}-bound) form. (However, these investigators also demonstrated that simply increasing the Tris concentration of the buffer had a similar effect.) Therefore, we have examined the effects of Na^+ on the secondary structure of type III (calcium-depleted) α-lactalbumin. Table III lists the peak positions and relative integrated intensities of α-lactalbumin in the presence of two different concentrations of NaCl, 40 and 200 mM. The spectrum collected in the presence of 40 mM Na^+ appears essentially identical with the spectrum of the apo (Ca^{2+}-free) form of α-lactalbumin, although small changes, similar to those observed upon Mn^{2+} binding, are indeed observed. This result is in agreement with the aromatic CD studies cited above which indicate little difference in the CD spectra of α-lactalbumin at low Na^+ concentrations. However, the infrared spectrum collected in the presence 200 mM Na^+ still essentially resembles the spectrum of apo α-lactalbumin, although the extent of the changes observed are somewhat greater for the higher concentration of sodium. Thus, a transition to the holo form, as characterized by a complete loss of the 1646 cm^{-1} component with concomitant increases in the contribution of bands arising from helical structures, is not observed at the high Na^+ concentration. It is possible that these spectral changes which occur with added Na^+ simply reflect minor conformational changes associated with the increased ionic strength because Hiroaka and Sugai (1985) have demonstrated changes with increasing ionic strength by increasing the Tris concentration. In summary, infrared spectroscopic results indicate that although Na^+ binding induces small conformational changes, it does not shift the backbone conformation of α-lactalbumin to nearly the same extent as the Ca^{2+} ion, as was suggested by the aromatic CD studies (Hiroaka & Sugai, 1985).

The infrared results of Na^+-binding experiments further support the argument that the fluorescence and aromatic CD changes observed on binding of ions to the Ca^{2+} site are due in large part to the presence of the charged species and are not due to secondary structural changes induced on binding. As was observed upon binding of Mn^{2+}, only small secondary structure differences are observed with infrared spectroscopy, although large-scale changes in fluorescence are observed, similar to that of Ca^{2+} binding.

Binding of Zinc. Previous studies have indicated that Zn^{2+} (r_{Zn} = 74 pm; Shannon & Previtt, 1969) also binds to α-lactalbumin but at a different site from that of Ca^{2+} (Murakami & Berliner, 1983). Initial studies have also suggested that binding of Zn^{2+} to α-lactalbumin which has already bound Ca^{2+} results in displacement of the Ca^{2+}, with a concomitant change in the conformation to a conformer which resembles, based on fluorescence quenching studies, the apo form of α-lactalbumin. A conformational change was proposed by these investigators for the mechanism of this Zn^{2+}-induced calcium displacement. However, later studies have indicated that Ca^{2+} and Zn^{2+} can bind to α-lactalbumin simultaneously but that the Ca^{2+}- and Zn^{2+}-bound protein resembles the apo form (Musci & Berliner, 1985a,b). No changes in fluorescence quenching were observed for the Zn^{2+} binding to apo α-lactalbumin in this study.

Table III lists the peak positions and relative intensities of the deconvoluted amide I′ region of the spectrum of α-lactalbumin (type III, Ca^{2+}-depleted) to which 3 mM Zn^{2+} alone had been loaded (higher concentrations of Zn^{2+} resulted in precipitation of the protein). Small but significant differences are apparent between this spectrum and that of the apo form where no metal ions are present. The most apparent change is the appearance of a band near 1622 cm^{-1} upon loading of Zn^{2+} concomitant with an equivalent loss of the relative integrated intensity from the component band near 1646 cm^{-1}. The remainder of the spectrum remains essentially as with the apo form. These conformational changes may not be a result of the Zn^{2+} binding, per se, but possibly from aggregation induced by Zn^{2+} as previous studies have suggested that bands near 1622 cm^{-1} are associated with the formation of intermolecular β-sheet complexes (Casal et al., 1989; Byler & Purcell, 1989). Thus, from these results, it would appear that binding of Zn^{2+} to apo α-lactalbumin induces little secondary structural change, suggesting a rigid binding site.

Table III also lists the relative intensities and peak positions of the amide I′ region of apo α-lactalbumin previously loaded

with Ca^{2+} (10 mM) and then loaded with Zn^{2+} (2 mM; higher concentrations of Zn^{2+} resulted in precipitation of the protein). The observed infrared spectrum is essentially identical with that found for the holo form of α-lactalbumin (i.e., Ca^{2+} bound only). While the ratio of Zn^{2+} to protein in this experiment is slightly less than stoichiometric, given the large scale of the conformational transition between the holo and apo forms of this protein, we would expect to observe measurable infrared spectral differences even for a fraction of protein molecules in the apo form. No such change is observed in either the deconvoluted spectra or the second-derivative spectra (not shown). Thus, these results indicate that binding of Zn^{2+} to holo α-lactalbumin does not result in a backbone conformational change to an apolike conformer. Rather, they appear similar to results found for binding of Ca^{2+} to the Zn^{2+} site. For both cases, Ca^{2+} or Zn^{2+} binding to the Zn^{2+}-binding site, the infrared spectra resemble the holo form while the fluorescence characteristics resemble those of the apo form.

It would appear from the results presented here that some of the information concerning the conformation of α-lactalbumin from these infrared studies differs from that of other studies. However, it is important to examine possible effects which are monitored by fluorescence and aromatic circular dichroism studies and compare these with infrared spectroscopic data. Both fluorescence and aromatic circular dichroism studies are sensitive to the local environment, solvent exposure, tertiary structure, electrostatics, and conformation of the aromatic residues which give rise to their respective properties. In contrast, the amide I region of infrared spectra is predominantly sensitive to the protein's backbone conformation. Thus, these different spectroscopic experiments provide complementary information. With the additional data now available from infrared spectroscopic studies, models for metal ion binding to α-lactalbumin can be further refined.

Conclusion

Our studies of the secondary structure of α-lactalbumin using resolution-enhanced infrared spectroscopy have provided important new information concerning the conformational states of this complex molecule in the presence of various metal ions. The experimental results reported here reveal that removal of Ca^{2+} from the holo form of the native protein induces a large, distinct change in the secondary structure, most likely in the Ca^{2+}-binding loop. By contrast, none of the other ions studied appears able to stabilize a conformation similar to that of the holo α-lactalbumin (Ca^{2+}-bound). Nor does binding of Zn^{2+} or Ca^{2+} to the Zn^{2+} site of the holo α-lactalbumin form induce a conformational change, of the sort which was suggested by fluorescence experiments, that can be detected by infrared spectroscopy. Clearly, the present results suggest that current models of the metal-binding properties of α-lactalbumin need to be carefully reexamined in light of these findings. This new information may also be helpful in interpretation of structure–function studies in which the physiological role of these ions with respect to α-lactalbumin is examined.

Acknowledgments

We thank Ms. Ellen Smuda for her help in collection of the infrared data and in preparation of the figures, Mr. Michael Kurantz for supplying the atomic absorption data, Ms. Dorothy Brower for providing the electrophoresis information, Dr. Lawrence Berliner for help with experimental protocols and for critically reviewing an earlier version of the manuscript, and Drs. Thomas Abbott and Eugene Permyakov for additional discussion of the manuscript.

References

Acharya, K. R., Stuart, D. I., Walker, N. P. C., Lewis, M., & Phillips, D. C. (1989) *J. Mol. Biol. 208*, 99–127.

Bano, M., Salomon, D., & Kidwell, W. R. (1985) *J. Biol. Chem. 260*, 5745–5752.

Berliner, L. J., & Johnson, J. D. (1988) in *Calcium Binding Proteins* (Thompson, M. P., Ed.) Vol. 2, pp 79–116, CRC Press, Boca Raton, FL.

Brodbeck, U., Denton, W. L., Tanahashi, N., & Ebner, K. E. (1967) *J. Biol. Chem. 242*, 1391–1397.

Byler, D. M., & Susi, H. (1986) *Biopolymers 25*, 469–487.

Byler, D. M., & Susi, H. (1988) *J. Ind. Microbiol. 3*, 73–88.

Byler, D. M., & Purcell, J. M. (1989) *Spectrosc. Biol. Mol., Proc. Eur. Conf., 3rd*, 21–24.

Casal, H. L., Kohler, U., & Mantsch, H. H. (1989) *Biochim. Biophys. Acta 957*, 11–20.

Chirgadze, Y. N., Fedorov, O. V., & Trushina, N. P. (1975) *Biopolymers 14*, 679–694.

Covington, A. K., Paabo, M., Robinson, R. A., & Bates, R. G. (1968) *Anal. Chem. 40*, 700–706.

Desmet, J., & van Cauwelaert, F. (1988) *Biochim. Biophys. Acta 957*, 411–419.

Desmet, J., Hanssens, I., & van Cauwelaert, F. (1987) *Biochim. Biophys. Acta 912*, 211–219.

Dong, A., Huang, P., & Caughey, W. S. (1990) *Biochemistry 29*, 3303–3308.

Dousseau, F., & Pezolet, M. (1990) *Biochemistry 29*, 8771–8779.

Goormaghtigh, E., Cabiaux, V., & Ruysschaert, J.-M. (1990) *Eur. J. Biochem. 193*, 409–420.

Haris, P. I., Lee, D. C., & Chapman, D. (1986) *Biochim. Biophys. Acta 874*, 255–265.

Hiraoka, Y., & Sugai, S. (1985) *Int. J. Pept. Protein Res. 26*, 252–261.

Holloway, P., & Mantsch, H. H. (1989) *Biochemistry 28*, 931–935.

Kauppinen, J. K., Moffatt, D. J., Mantsch, H. H., & Cameron, D. C. (1981) *Appl. Spectrosc. 35*, 271–277.

Krimm, S., & Bandekar, J. (1986) *Adv. Protein Chem. 38*, 181–364.

Kronman, M. J., Sinha, S. K., & Brew, K. (1981) *J. Biol. Chem. 256*, 8582–8587.

Kuwajima, K., Harushima, Y., & Sugai, S. (1986) *Int. J. Pept. Protein Res. 27*, 18–27.

Lee, D. C., & Chapman, D. (1986) *Biosci. Rep. 6*, 235–255.

Lee, D. C., Haris, P. I., Chapman, D., & Mitchell, R. C. (1990) *Biochemistry 29*, 9185–9193.

Miyazawa, T., & Blout, E. R. (1961) *J. Am. Chem. Soc. 83*, 712–719.

Murakami, K., & Berliner, L. J. (1983) *Biochemistry 22*, 3370–3374.

Murakami, K., Andree, P. J., & Berliner, L. J. (1982) *Biochemistry 21*, 5488–5494.

Musci, G., & Berliner, L. J. (1985a) *Biochemistry 24*, 3852–3856.

Musci, G., & Berliner, L. J. (1985b) *Biochemistry 24*, 6945–6948.

Olinger, J. M., Hill, D. M., Jakobsen, R. J., & Brody, R. S. (1986) *Biochim. Biophys. Acta 869*, 89–98.

Permyakov, E. A., Yarmolenko, V. V., Kalinichenko, L. P., Morozova, L. A., & Burstein, E. A. (1981a) *Biochem. Biophys. Res. Commun. 100*, 191–197.

Permyakov, E. A., Kalinichenko, L. P., Morozova, L. A., Yarmolenko, V. V., & Burstein, E. A. (1981b) *Biochem. Biophys. Res. Commun. 102*, 1–7.

Permyakov, E. A., Morozova, L. A., & Burstein, E. A. (1985) *Biophys. Chem. 21*, 21-31.
Prestrelski, S. J., Byler, D. M., & Thompson, M. P. (1991a) *Int. J. Pept. Protein Res. 37*, 508-512.
Prestrelski, S. J., Byler, D. M., & Liebman, M. N. (1991b) *Biochemistry 30*, 133-143.
Segawa, T., & Sugai, S. (1983) *J. Biochem. 93*, 1321-1328.
Shannon, R. D., & Previtt, C. T. (1969) *Acta Crystallogr. B25*, 925-946.
Stuart, D. I., Acharya, K. R., Walker, N. P. C., Smith, S. G., Lewis, M., & Phillips, D. C. (1986) *Nature 324*, 84-87.
Surewicz, W. K., & Mantsch, H. H. (1988) *Biochim. Biophys. Acta 952*, 115-130.
Susi, H. (1969) in *Structure and Stability of Biological Macromolecules* (Timasheff, S. N., & Fasman, G. D., Eds.) pp 576-663, Marcel Dekker, New York.
Susi, H., & Byler, D. M. (1983) *Biochem. Biophys. Res. Commun. 115*, 391-397.
Susi, H., & Byler, D. M. (1986) *Methods Enzymol. 130*, 290-311.
Trewhella, J., Liddle, W. K., Heidorn, D. B., & Strynadka, N (1989) *Biochemistry 28*, 1294-1301.

Raman Spectroscopic Studies of NAD Coenzymes Bound to Malate Dehydrogenases by Difference Techniques†

Hua Deng,‡ John Burgner,*,§ and Robert Callender*,‡

Physics Department, City College of the City University of New York, New York, New York 10031, and Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Received April 5, 1991; Revised Manuscript Received June 26, 1991

ABSTRACT: We report here on the Raman spectra of NADH, 3-acetylpyridine adenine dinucleotide, $APAD^+$, and a fragment of these molecules, adenosine 5′-diphosphate ribose (ADPR) bound to the mitochondrial (mMDH) and cytoplasmic (or soluble, sMDH) forms of malate dehydrogenase. We observe changes in the Raman spectrum of the adenosine moiety of these cofactors upon binding to mMDH, indicating that the binding site is hydrophobic. On the other hand, there is little change in the spectrum of the adenosine moiety when it binds to sMDH. Such observations are in clear contrast with those results obtained in LDH and LADH, where there are significant changes in the spectrum of the adenosine moiety when it binds to these two proteins. A strong hydrogen bond is postulated to exist between amide carbonyl group of NAD^+ and the enzyme in the binary complexes with both mMDH and sMDH on the basis of a sizable decrease in the frequency of the carbonyl double bond. The interaction energy for formation of a hydrogen bond is the same as found previously for LDH, and we estimate that it is 2.8 kcal/mol more favorable in the binary complex than in water. A hydrogen bond is also detected between the amide $-NH_2$ group of NADH and sMDH that is stronger than that formed in water and is of the same size as found in LDH. Surprisingly, the hydrogen bond to the $-NH_2$ group in mMDH is the same as that found for water. The strength of these hydrogen bonds likely contributes to the varying observed λ_{max} shifts in the near-UV absorbance of the reduced nicotinamide when it binds to these enzymes. The lack of increased hydrogen-bond strength in the binary complex of NADH with mMDH relative to that found in water is proposed as a partial explanation of the red-shift in λ_{max} found for reduced binary complex with mMDH, which is unusual for an A-side dehydrogenase. On the basis of the strength of the observed hydrogen-bond interactions between the acetyl moiety of $APAD^+$ and the enzyme in the binary complex, we estimate that the energy difference between the syn and anti conformers of the pyridinium ring of the bound cofactor is at least 7.3 kcal/mol in mMDH and substantially more than this in sMDH and LDH where stronger interactions with the amide $-NH_2$ can occur. This energy difference is sufficient, or nearly so, to determine the high degree of stereospecificity that is observed with these enzymes during transfer the *pro-R* hydrogen from NADH to substrate (LaReau et al., 1989).

To further our understanding of the changes in the bond orders of cofactors and substrates when they are transferred from the solvent to the more restrictive environment in the active site of a dehydrogenase, we extended our studies using sensitive Raman difference spectroscopy begun with ADH[1] and LDH (Callender et al., 1988; Chen et al., 1987; Deng et al., 1989a,b; Yue et al., 1984) to the mitochondrial and cytosolic forms of malate dehydrogenase. The Raman spectrum of the bound molecule is obtained by measuring the spectrum

† This work was supported by Grants GM35183 (City College) and G12 RR03060 (City College) from the National Institutes of Health and Grant 8616216 from the National Science Foundation (Purdue University).
‡ City College of the City University of New York.
§ Purdue University.

[1] Abbreviations: NADH, reduced β-nicotinamide adenine dinucleotide; mMDH, mitochondrial malate dehydrogenase; sMDH, cytoplasmic malate dehydrogenase; ADPR, adenosine 5′-diphosphate ribose; $APAD^+$, oxidized acetylpyridine adenine dinucleotide; OMA, optical multichannel analyzer; LADH, liver alcohol dehydrogenase; LDH, lactate dehydrogenase.

0006-2960/91/0430-8804$02.50/0