Infrared Spectroscopic Study of the pH-Dependent Secondary Structure of Brain Clathrin†

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ABSTRACT: The lattice material surrounding brain coated vesicles is formed predominately from a M_r 180000 polypeptide subunit structure termed clathrin. The vesicle coat is dissociated from the bilayer membrane by mild alkaline solutions (pH 8.5) to form clathrin trimers, or triskelions. In solution, at pH 6.5, the triskelions assemble into cagelike structures exhibiting lattices whose hexagonal and pentagonal polyhedral faces are similar to those associated with the coated vesicles. The secondary structures of these clathrin species in aqueous media were characterized through an examination of the infrared spectroscopic amide I and II vibrational frequency regions in an effort to monitor the structural changes that arise as the water-soluble triskelions assemble into cagelike networks. The reassembly process is accompanied by an increase in the peak height intensity ratio of the amide II to amide I region from 0.38 to 0.68 and a shift in the amide I frequency from 1648 to 1656 cm^{-1}. These spectral changes indicate a decrease in the α-helical content of the triskelion polypeptide chains as they assemble to form cages. Other spectral features associated with the amide I and II bands indicate the presence of both β-sheet and β-turn protein conformations in the clathrin structures. The amide I and II frequency and intensity characteristics reflecting the clathrin coat of intact vesicles are nearly identical with analogous spectra of the clathrin cages reassembled from triskelions in the absence of membrane components. The spectral results suggest that for structural studies involving protein–protein and protein–lipid interactions, the reconstituted, water-soluble cages provide faithful model systems for the clathrin coat associated with intact membranes.

Coated vesicles, subcellular organelles ubiquitous to almost all eukaryotic cells, have been implicated in a variety of shuttling mechanisms including endocytic (Goldstein et al., 1979; Salisbury et al., 1980; Fan et al., 1982) as well as secretory processes (Franke et al., 1976; Rothman & Fine, 1980). Although coated vesicles have been isolated from a number of tissue sources, they all exhibit a remarkable lattice of rodlike structures surrounding the surface of the membrane bilayer. The basic unit of the vesicle coat is the triskelion, a three-legged trimer consisting of three M_r 180000 polypeptides associated with three additional polypeptide subunits of M_r ~35000 (Pearse, 1975; Pretorius et al., 1981; Ungewickell & Branton, 1981; Lisanti et al., 1982). The three 445-Å legs, bent at approximately 190 Å from a common vertex, interact with neighboring units to form the characteristic polyhedral coat material around the vesicle (Ungewickell & Branton, 1981; Slayter, 1982; Kanaseki & Kadota, 1969; Crowther et al., 1976).

Under defined conditions, dissociated clathrin triskelions reassemble in the absence of a vesicle membrane into cagelike structures resembling the surface lattice of coated vesicles (Keen et al., 1979; Woodward & Roth, 1979; Nandi et al., 1980). Using this model system, Crowther & Pearse (1981) recently examined modes of packing triskelion units into cagelike forms. These authors proposed a system in which the clathrin molecule represents a relatively stiff assembly exhibiting a variable joint at the vertex of the triskelion and a hinge within the legs at approximately 160 Å from the common junction.

In monitoring the conformational reorganizations paralleling the evolution of triskelions into high molecular weight species, reassemblied units have been investigated by circular dichroism. Although circular dichroism spectra of cages are subject to light scattering artifacts, the technique suggests that for triskelion units α-helical structures account for approximately 50% of the peptide backbone (Pretorius et al., 1981; Ungewickell et al., 1982; Steer et al., 1982). In order to elucidate further the secondary structures available to clathrin, we have applied infrared procedures in the present paper toward examining the vibrational spectra of triskelions, isolated from bovine brain tissue, in their solution and reassembled states.

Infrared spectroscopy is an established technique for qualitatively identifying the secondary structures formed by proteins and polypeptides. In deducing structural features from infrared data, the characteristic vibrational frequencies of the trans CONH amide moiety of the macromolecular assembly are specifically investigated (Thomas & Kyogoku, 1977; Fawcett & Long, 1973; Susi, 1969). Not all of the amide vibrational modes are experimentally accessible because of either solvent interference, weak band intensity, or instrumental considerations. As a consequence, in this report we will be concerned only with the amide I and II modes occurring in the 1700–1500-cm^{-1} spectral region. The amide I band, located at approximately 1650 cm^{-1}, is assigned to a C=O stretching vibration coupled weakly to the CN stretching and the in-plane NH bending modes (Thomas & Kyogoku, 1977; Susi, 1969). Appearing generally in the 1575–1480-cm^{-1} region, the amide II band is assigned to a C–N distortion that is strongly coupled to the in-plane N–H bending vibration of the peptide linkage (Thomas & Kyogoku, 1977; Susi, 1969). The spectral characteristics of the amide I and II vibrational bands are sensitive to the forms of secondary structure that involve specific hydrogen bonds between the carbonyl oxygen and N–H hydrogen atoms either from units in the same peptide chain or from other chains, as, for example, in α-helical and β-sheet structures, respectively.

For biological systems, structures of interest often occur in aqueous media, a situation that complicates the observation of infrared spectra particularly in the amide I region around 1650 cm^{-1}. Since the HOH bending mode of water strongly

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absorbs infrared radiation at about 1640 cm^{-1}, this feature must be suitably subtracted from the protein solution spectrum before the amide I and II bands at 1650 and 1550 cm^{-1} are revealed in a meaningful manner. In attempts to circumvent the interference of water in this spectral region, investigators have replaced the solvent with ^2H_2O, a system whose deformation mode is shifted to lower infrared frequencies (Schnarr & Maurizot, 1980; Rothschild et al., 1980; Osborne & Nabedryk-Viala, 1978). Unfortunately, solvent interference remains a problem even under these conditions because of contributions from HOD species. Further, the ready exchange of the amide hydrogen by deuterium leads to shifts in the amide I and II frequencies. Since carefully prescribed infrared spectroscopic techniques, involving either dispersive instruments or interferometers, permit the reliable subtraction of either the solvent medium or other constituents in a multicomponent system, we chose H_2O as the more relevant solvent for the protein assemblies described in this report.

Experimental Procedures

Isolation of Coated Vesicles and Clathrin Triskelions. Coated vesicles were isolated from bovine brain according to the method of Nandi et al. (1982). In a typical preparation performed at 4 °C, five bovine brains, immediately chilled on ice after slaughter, were cleaned of remaining meninges, pia, and white matter. Approximately 1400 of gray matter was homogenized with an equal volume of buffer containing 0.1 M Mes,[1] pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.02% NaN_3. The homogenate was centrifuged for 45 min at 10000 rpm (16000*g*) in a GSA rotor; the resulting supernatant was centrifuged at 34000 rpm (100000*g*) in a Beckman 35 rotor for 60 min. Each pellet was resuspended, pooled, and then centrifuged at 10000 rpm (12000*g*) in an SS-34 rotor to remove aggregated material. The supernatants were combined, diluted 1:1 in homogenization buffer, and centrifuged for 60 min at 37000 rpm (130000*g*) in a Beckman 45 Ti rotor. After the supernatants were discarded, each pellet was resuspended as described above, pooled, centrifuged at 12000*g* for 10 min, diluted 1:1 with buffer, and then centrifuged a second time at 37000 rpm. The supernatnats were discarded, and the pellets were resuspended in 80 mL of homogenization buffer. After a slow-speed centrifugation to remove particulate material, 6-mL aliquots were layered onto 6 mL of an 8% sucrose/D_2O solution maintained at the appropriate pH with the same buffer salts as in the homogenziation buffer. The single-step gradients were centrifuged for 2 h at 32000 rpm (125000*g*) in an SW 40 rotor at 16 °C. The supernatants were aspirated, and the pellets were washed several times with buffer. Pellets could be stored at 4 °C for several weeks without a change in either composition or structure of the coated vesicle. When purified coated vesicles were needed, the pellets were resuspended in 4–5 mL of 0.1 M Mes buffer at pH 6.5 and centrifuged at 12000*g* for 10 min. The supernatant provided a greater than 95% pure source of coated vesicles as determined by electron microscopy (A. C. Steven, J. F. Hainfield, J. S. Wall, and C. J. Steer, unpublished results).

Clathrin trimers (triskelions) were isolated by dialyzing the coated vesicles at 4 °C overnight against two 1-L changes of buffer containing 10 mM Tris-HCl, pH 8.5. The uncoated vesicles were pelleted by centrifuging at 50000 rpm (170000*g*) in a Beckman 65 rotor for 60 min. In general a single pellet of coated vesicles yielded about 4–5 mg of protein. NaDodSO_4–PAGE of the supernatant revealed the characteristic pattern of the M_r 180000 clathrin band together with the clathrin associated proteins at 33000 and 36000 daltons (data not shown). In order to decrease the Tris-HCl concentration, the solutions of clathrin trimers were dialyzed overnight against 1 mM Tris-HCl, at pH 8.5. The material was then pooled to a total volume of approximately 20–25 mL containing 12–18 mg of protein as determined by the absorbance at 280 nm by using a specific absorptivity $E^{1\%}_{1\,cm}$ of 10.0 (Kirchhausen & Harrison, 1981). After quickly freezing in solid carbon dioxide, the material was lyophilized overnight and resolubilized in 100–150 μL of buffer containing 100 mM Tris-HCl, pH 8.5. Insoluble material was pelleted by centrifuging the sample for 10 min at 100000*g*. Usual preparations of the clathrin trimers yielded concentrations in the range of 75–125 mg/mL as determined by solution absorption at 280 nm and Lowry protein assays (Lowry et al., 1951).

Samples of triskelions to be examined in the lyophilized state were dialyzed further against distilled water brought to pH 8.5 with NaOH. After lyophilization, the samples, completely devoid of Tris-HCl, were stored at 4 °C.

The uncoated vesicles, recovered as described above, were gently resuspended in 3–4 mL of 10 mM Tris-HCl, pH 8.5, and dialyzed overnight a second time against two 1-L changes of similar buffer. After high-speed centrifugation, 100 mL of 0.1 M Tris-HCl, pH 8.5, was layered over the pellet of uncoated vesicles. NaDodSO_4–PAGE of the sample revealed that greater than 95% of the clathrin band had been dissociated from the vesicle membrane (data not shown).

Reassembly of the triskelions into re-formed clathrin cages was accomplished by dialyzing the clathrin trimers (approximately 0.5 mg/mL concentration in 10 mM Tris-HCl, pH 8.5) against two 1-L changes of homogenization buffer, pH 6.5. Similar results were obtained whether the clathrin was dialyzed immediately after dissociation from the coated vesicles or after lyophilization concentration and dilution. The results suggested that these latter steps had no adverse affect on the clathrin trimers' ability to reassemble into cages. NaDodSO_4–PAGE revealed characteristic bands at M_r 180000, 33000, and 36000. Cages were pelleted at 50000 rpm in the Beckman 65 rotor for 1 h. A total of 200 μL of 0.1 M Mes buffer, pH 6.5, was layered over the pellets to keep them hydrated.

Infrared Spectroscopic Measurements. Infrared spectra were obtained with a Perkin-Elmer 580 B spectrophotometer controlled by the National Institutes of Health LDACS (Laboratory Data Acquisition Systems) computer. LDACS consists of a local LSI-11 based computer with a Tektronix 4006 graphics terminal for communicating with a Laboratory PDP 11-70 computer for data storage and manipulation. Since the infrared spectra of protein solutions generally involve broad bands, spectra were observed under moderate resolution conditions utilizing spectral slit widths of the order of 1.8–2.5 cm^{-1}. Data points were collected over the range of 2200–1200 cm^{-1} for each spectrum. A jacketed variable path-length cell with CaF_2 windows was thermostated at 21 °C for all recorded spectra, except those involving fluorolube dispersions. In general, a small amount of the hydrated, pelleted material, together with 1 or 2 drops of an appropriate buffer solution, was placed in the center of one window of the disassembled cell; the other window was then lowered until the sample formed a uniform, bubble-free film between the CaF_2 plates.

[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrogen chloride; NaDodSO_4–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

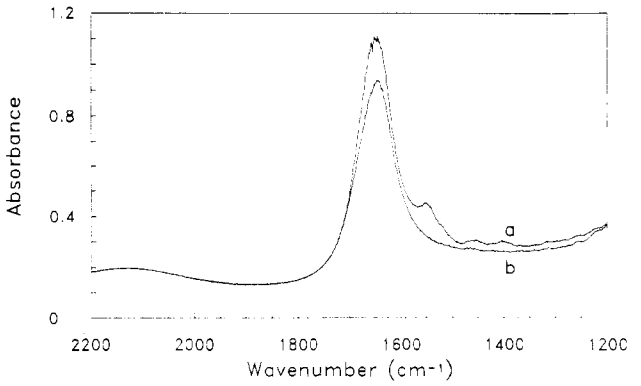

FIGURE 1: (a) Spectrum of clathrin cages suspended in 0.1 M Mes buffer at pH 6.5. (b) Spectrum of 0.1 M Mes buffer solution. The reference buffer spectrum is matched to the protein spectrum by the 2130-cm^{-1} water-association band. The resulting subtracted spectrum for the clathrin cages is given in Figure 4a.

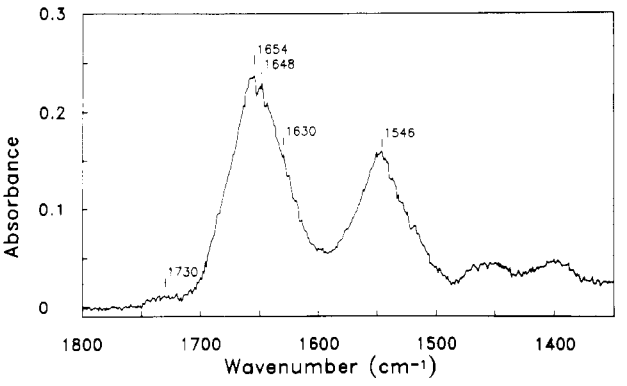

FIGURE 2: Infrared spectra of bovine brain coated vesicles in the amide I and II region at 21 °C in 0.1 M Mes buffer at pH 6.5. Spectral contributions from water have been subtracted.

Spectra were recorded and stored for several path lengths, ranging from 10 to 3 μm. The instrument was continuously purged of water vapor with dry nitrogen gas. Water vapor lines were used for wavelength calibration; vibrational frequencies for defined peaks are reported to within ± 1–2 cm^{-1}, although greater uncertainties probably exist for the shoulder features. For samples containing lyophilized clathrin suspended in fluorolube, the dispersions were placed between NaCl windows and mounted in the beam at ambient temperature.

Spectra of the buffer solutions, Mes and Tris-HCl, without protein were also recorded in the variable path-length cell at 21 °C. The path length was decreased in small steps in order to develop extensive libraries for solvent spectra over the 10–3-μm range. In solution, the protein amide I and II vibrational bands are strongly overlapped with the intense water deformation band at approximately 1640 cm^{-1}. The intensities and frequencies of the underlying amide I modes depend critically upon the employed computer subtraction procedures. We utilized the water association band absorbance at approximately 2130 cm^{-1} in the clathrin solutions for determining the appropriate buffer library spectrum to be used in subtracting the water features from the spectrum of the solute. Figure 1 displays a typical spectrum of the protein, as, for example, clathrin cages, in buffer (Figure 1a). Figure 1b displays the appropriate buffer solution and its spectral match in the 2130-cm^{-1} water band region. Figure 4a presents the resulting difference spectrum for the clathrin cages. We emphasize that although the final amide I and II band infrared spectra were smoothed with a seven point cubic least-squares convolution algorithm for figure presentation, no spectral distortions were introduced by this routine. We note that although the expanded spectra displayed in Figures 2–6 appear to contain noise, we elect to present these unbiased spectra over spectra that often prove to be excessively smoothed. The features listed in Table I were shown to be reproducible for separate clathrin preparations. Features attributed both the shoulders and to prominent peaks also appear reproducibly in spectra subjected to a variety of smoothing algorithms (data not shown).

Infrared spectra of strongly absorbing solutions observed in short path-length cells may have distorted line shapes resulting from possible interference patterns and from changes in the reflectance at the solution–cell window interface. The latter reflectance effect is caused by a variation in the index of refraction of a strong solution absorption band (Fujiyama et al., 1970). In attempting to assess these effects on the amide I and II region spectra, we followed the above procedures for subtracting the water spectrum for a number of samples containing various solute concentrations. The resulting protein spectra in the amide I and II regions were similar in relative intensities and peak frequencies, indicating that reproducible infrared spectra may be obtained with short path-length cells by following the procedures described above. For the systems examined in the present study, variations of the index of refraction through the absorption bands do not lead to significant band distortions. While we contend that extremely short path-length cells are amenable to recording reliable spectra representative of components present in dilute aqueous solutions, we emphasize the necessity of matching approrpiate library reference spectra in the spectral subtraction steps.

Results and Discussion

The water-subtracted infrared spectra of clathrin related systems were recorded for the following species: (a) coated vesicles in Mes buffer at pH 6.5, (b) uncoated vesicles, that is, vesicles with the clathrin coat removed in Tris-HCl buffer at pH 8.5, (c) clathrin cages, namely, clathrin coats reconstituted at pH 6.5 in Mes buffer in the absence of vesicle structures, (d) clathrin triskelions, the dissociated clathrin coat, in Tris-HCl buffer at pH 8.5, and (e) lyophilized clathrin. In the following sections we will examine the amide I and II absorption regions at approximately 1650 and 1545 cm^{-1}, respectively, in an effort to associate the observed spectral features with specific conformational species. The identification of conformational structures from the behavior of the vibrational spectra is based upon on abundance of spectral information available from extensive frequency compilations and from numerous correlations derived from detailed normal coordinate analyses (see, for example, Thomas & Kyogoku, 1977; Fawcett & Long, 1973; Koenig, 1972; Krimm & Bandekar, 1980; Susi, 1969, and references cited therein).

(*A*) *Coated Vesicles.* Figure 2 illustrates the amide I and II regions of the water-subtracted infrared spectrum of the coated vesicles at 21 °C. Distinct, reproducible spectral peaks are observed at 1654 and 1648 cm^{-1} in the amide I region and at 1546 cm^{-1} in the amide II region. A number of reproducible shoulders, associated with the primary features, are tabulated and assigned in Table I. The absorption at approximately 1730 cm^{-1} is assigned to the carbonyl stretching modes arising from the vesicle bilayer lipid. The peaks at 1648 and 1546 cm^{-1} are characteristic of α-helical structures, while the 1654-cm^{-1} maximum suggests unordered structures (Thomas & Kyogoku, 1977; Susi, 1969). The presence of β-structures and β-turns is also indicated in the spectrum (Thomas & Kyogoku, 1977; Susi, 1969; Moore & Krimm, 1976a,b; Krimm & Bandekar, 1980; Bandekar & Krimm, 1980). The ratio

Table I: Observed Infrared Amide I and II Frequencies and Intensity Ratios for Clathrin in Various Forms

sample	medium	temp (°C)	amide I frequency (cm^{-1})	structure assignment	amide II frequency (cm^{-1})	structure assignment	A_{II}/A_I[a]
coated vesicles	pH 6.5, Mes	21			1563 (sh)[b]		0.67
			1654	unordered			
			1648	α-helix	1546	α-helix	
			1644 (sh)	β-turns (?)	1537 (sh)		
			1630 (sh)	β-sheet	1530 (sh)	β-sheet	
uncoated vesicles	pH 8.5, Tris	21	1665 (sh)		1561 (sh)		0.67
			1655	unordered			
			1645 (sh)	α-helix	1548	α-helix	
					1538 (sh)		
cages	pH 6.5, Mes	21			~1580 (sh)	β-turns	0.68
			1666 (sh)		1561 (sh)		
			1657	unordered	1550	α-helix	
			1651	α-helix			
			1644 (sh)				
			1638 (sh)		1530 (sh)	β-sheet	
			~1630 (sh)	β-sheet			
triskelions	pH 8.5, Tris	21	~1685 (sh)	β-sheet	~1570 (filling in)	unordered	0.38
			1676 (sh)				
			1657	unordered	1545 (br)	α-helix	
			1646	α-helix			
			~1631 (sh)	β-sheet			
clathrin (lyophilized)	Fluorolube	ambient	1679 (sh)	β-sheet			0.70
					1545	α-helix	
			1656	unordered	1537	β-sheet	
			1649 (sh)	α-helix	1518 (sh)	unordered	
			1638 (sh)	β-sheet			

[a] A_{II}/A_I represents the peak height intensity ratio between the amide I and amide II vibrational transitions. [b] sh represents a reproducible shoulder or inflection on the absorption peak as deduced from separate clathrin preparations. [A variety of noise smoothing techniques (data not presented) were consistent with the determination of shoulders based upon spectra from multiple preparations.]

of the amide II to amide I absorbance maxima (A_{II}/A_I) is 0.67. In addition to the frequency data Table I also summarizes the intensity information for the various clathrin species. The spectral characteristics noted for the coated vesicles represent a superposition of the infrared absorptions from the clathrin coat material as well as the integral and peripheral protein constituents associated with the membrane bilayer.

(B) Uncoated Vesicles. As discussed above, clathrin is dissociated from the coated vesicles by raising the pH to 8.5. Figure 3 presents the water-subtracted infrared spectrum of the uncoated vesicle pellet resuspended in buffer solution at 21 °C. Except for the 1730-cm^{-1} lipid carbonyl stretching mode of the uncoated vesicles being relatively more intense in comparison to the amide I and II bands, the uncoated vesicle spectrum appears quite similar to the coated vesicles spectrum. The spectrum, representative of membrane bilayer integral and peripheral protein components, indicates contributions from both α-helical and extended structures. The 1655-cm^{-1} maximum in the amide I band is consistent with random-coil conformations, while intensity in the 1645–1648-cm^{-1} region indicates α-helical contributions to the protein assembly. Although inflections in the spectral contours occur in the frequency regions expected for β-sheet and β-turn structures, it is difficult to unambiguously specify the conformations for the protein in the bilayer state. The intensity ratio A_{II}/A_I is 0.67, the same as that for the coated vesicle system. The similarities in the frequencies, half-widths, and A_{II}/A_I intensity ratios for the amide I and II regions of the spectra for the coated and uncoated vesicle material suggest that the general structural characteristics assumed by the clathrin coat and by the integral bilayer protein material are quite analogous. Without further knowledge of the integral, and possible peripheral, membrane constituents, it is difficult to assess the significance of the relationships between the two sets of spectra. A conservative estimate from the results of $NaDodSO_4$ gel procedures (data not shown) indicates that less than 10%

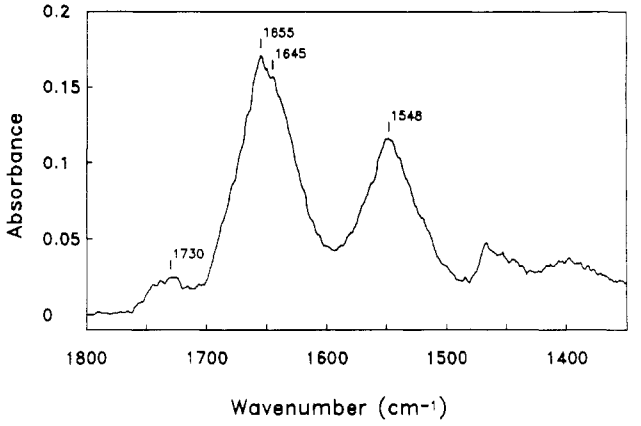

FIGURE 3: Infrared spectra of bovine brain uncoated vesicles in the amide I and II region at 21 °C in 0.1 M Tris-HCl buffer at pH 8.5. Spectral contributions from water have been subtracted.

clathrin is present in the uncoated vesicle preparations. Although it is difficult to estimate precisely the contribution of membrane protein to the amide region spectra for the coated vesicles in Figure 2, we judge on the basis of the bilayer lipid carbonyl signals at 1730 cm^{-1} (Figures 1 and 2) that approximately two-thirds to three-quarters of the signal for the coated vesicle spectrum arises from the clathrin coat. [In this comparison we assume that material contained within the vesicle interior is not present to significant levels (Whyte & Ockleford, 1980). Thus, the estimate is predicted upon the 1730-cm^{-1} absorption originating solely from bilayer lipid.]

For the uncoated vesicles, $NaDodSO_4$ gels show major components of approximately M_r 110 000 and 55 000. It is unclear as to whether these components are representative of solely integral membrane constituents or also reflect a peripheral bilayer protein species. Since these high molecular weight components are the predominant species present in uncoated vesicle assemblies, it is probably these constituents

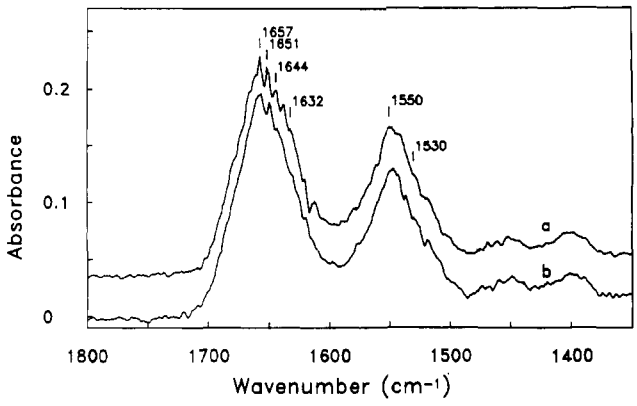

FIGURE 4: Infrared spectra of clathrin cages in the amide I and II region at 21 °C. (a) Spectra of reconstituted cages in 0.1 M Mes buffer at pH 6.5. (b) Spectra of the intact clathrin coat obtained by the subtraction of the uncoated vesicle spectrum from the coated vesicle spectrum after normalizing both spectra to the 1730-cm^{-1} lipid carbonyl stretching mode. Spectral contributions from water have been subtracted.

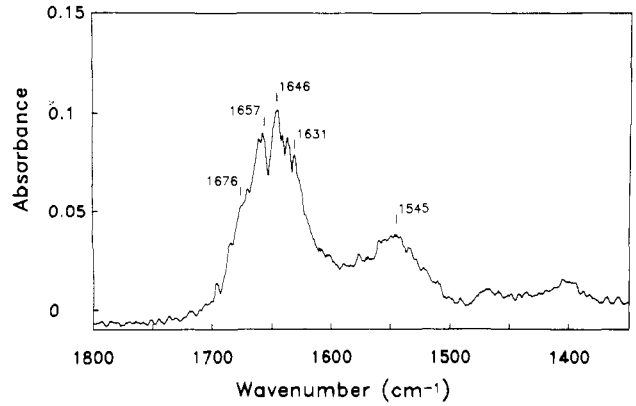

FIGURE 5: Infrared spectra of clathrin triskelions (~5 × 10^{-4} M) in the amide I and II region at 21 °C in 0.1 M Tris-HCl buffer at pH 8.5. Spectral contributions from water have been subtracted.

that are contributing secondary structures whose amide I and II spectra closely resemble those of vesicle-bound clathrin.

(*C*) *Clathrin Cages.* The spectra displayed in Figure 4 represent clathrin cages in two forms. Spectrum 4b was obtained by first normalizing both the coated vesicle and uncoated vesicle spectra, from which water was subtracted, to the lipid carbonyl stretching mode at approximately 1730 cm^{-1}. The spectrum for the uncoated system was subtracted from the coated vesicle spectrum. The resulting difference spectrum represents the amide I and II regions of the intact clathrin coat without contributions from the bilayer lipid or membrane proteins. This difference spectrum may be compared to the corresponding spectrum of the clathrin cages reassembled from triskelions at pH 6.5 in the absence of vesicle membranes. The spectrum of the reconstituted clathrin cages recorded at 21 °C, shown in Figure 4a, appears quite similar to the infrared spectrum of the intact clathrin coat in Figure 4b. The main features of these two spectra differ in peak frequencies by 1–2 cm^{-1}; the intensity ratio A_{II}/A_I = 0.68 is the same for both spectra. For the spectrum of the cages (Figure 4a), the 1657-cm^{-1} maximum indicates a considerable amount of unordered structures, while other features, listed in Table I, suggest, however, both α-helical and β-structure content. We conclude from the similarity of the two spectra in Figure 3 that the intact clathrin coat has nearly the same secondary structure as the cages reassembled from clathrin triskelions in the absence of bilayer systems. Thus, solutions of reconstituted clathrin cages should provide a conceptually simpler model system for investigating the structural and binding properties characteristic of the clathrin surrounding intact vesicles.

(*D*) *Clathrin Triskelions.* The amide I and II intervals for the water-subtracted infrared spectrum of clathrin triskelions at concentrations of approximately 5 × 10^{-4} M are shown in Figure 5. Infrared spectra of differing concentrations of clathrin cages and triskelions demonstrated that the spectral features of the dilute solutions of both species were the same as those for the more concentrated solutions. Spectra of the triskelions differ considerably from spectra of either the cages or coated vesicle assemblies. The maximum of the amide I feature shifts to 1646 cm^{-1}, indicating an increase in α-helical content for the triskelions. A second peak occurring at 1657 cm^{-1} reflects unordered conformations. The presence of α-helical structures for the triskelion forms at pH 8.5 is consistent with the determination of 50% α-helix by circular dichroism (Pretorius et al., 1981; Ungewickell et al., 1982; Steer et al., 1982). Furthermore, the amide A_{II}/A_I intensity ratio of 0.38 is reduced markedly from the 0.68 value representative of cages and coated vesicles. The triskelion spectrum also shows evidence of various β-structures (see Table I).

A precise interpretation of the peak height intensity ratios A_{II}/A_I obtained from the amide I and II spectral intervals is somewhat tenuous, particularly since the more relevant integrated band areas are difficult to obtain and since several conformational structures contribute intensity to a band interval. Further, the interpretation of experimental intensities in terms of molecular parameters becomes clouded as one must consider a variety of effects involving perturbations from the medium to both the charge reorganization and the attendant normal coordinate changes of the molecular vibrations under scrutiny. Despite these obstacles we may refer to empirical data that indicate that variations in the amide A_{II}/A_I intensity ratios are relevant in assessing changes in protein conformations. The observed decrease in A_{II}/A_I, going from 0.68 to 0.38 when clathrin cages transform to triskelions, is similar to the change in the same intensity ratio for the melittin/phospholipid system under conditions where α-helical conformations are presumably promoted. That is, the ratio A_{II}/A_I of the cleaved hydrophobic fragment F_1 (residues 1–19) of melittin in a lipid bilayer at 8 °C is 0.5 (Lavialle et al., 1982). The intensity ratio for the intact melittin system (residues 1–26) in reconstituted lipid bilayers at 13 °C is 0.75. The hydrophobic F_1 moiety of melittin probably exists in a predominantly α-helical conformation in either the cleaved fragment or intact polypeptide form. Intact melittin, however, also possesses a hydrophilic portion (residues 20–26) which assumes a variety of unordered or β-turn structures in addition to the α-helix formed by the hydrophobic moiety (residues 1–19) (Lavialle et al., 1982). The observed change in the intensity ratio suggests that an increase in the *relative* amount of α-helical content for the peptide structure results in a decrease in the amide A_{II}/A_I intensity ratio. This trend in the intensity ratio also occurs in a comparison between intact melittin and its hydrophobic fragment F_1 in 2-chloroethanol (Lavialle et al., 1982). Thus, the decrease in the A_{II}/A_I intensity ratio as clathrin cages dissociate to form triskelions parallels the conclusions arising from the frequency data in supporting a triskelion structure with an increased content of α-helical conformations.

(*E*) *Anhydrous Clathrin.* The amide I and II regions of the infrared spectrum of lyophilized clathrin suspended in a fluorocarbon (Fluorolube) mull are shown in Figure 6. As suggested in Table I, β-sheet and probable β-turn structures

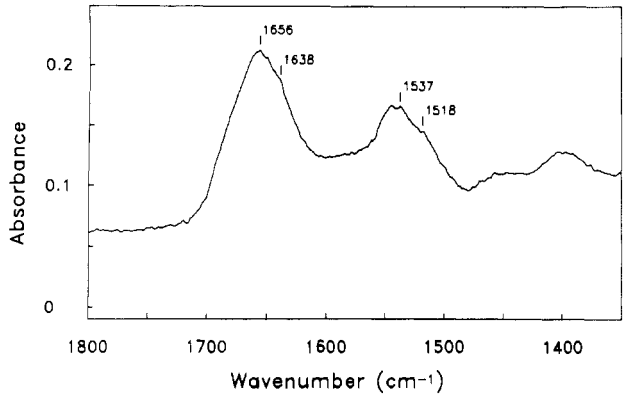

FIGURE 6: Infrared spectrum at room temperature of anhydrous clathrin in a fluorocarbon dispersion (Fluorolube).

exist in the lyophilized samples in addition to unordered and perhaps α-helical forms. It is interesting that despite the considerable filling in of intensity within the 1575–1625-cm^{-1} interval, the A_{II}/A_I peak height intensity remains 0.70, similar to the reassembled clathrin forms.

Conclusion

Although the assignment of the infrared active amide I and II spectral features to specific protein conformations provide, in general, qualitative notions of the secondary structures assumed by high molecular weight assemblies, the emerging ability to determine reliable protein spectra in the presence of strongly absorbing solvent bands significantly enhances the diagnostic potential of the vibrational technique. In particular, we have monitored the details of the infrared spectra of brain clathrin in the 1700–1500-cm^{-1} region as a probe of the structural rearrangements associated with this coat protein as it first dissociates from vesicular systems in aqueous solutions, forms characteristic three-legged triskelion moieties, and then reassembles into cagelike lattice networks in the absence of membrane components. A comparison between the conformationally sensitive amide I and II vibrational features for clathrin coated and uncoated vesicles suggests that the integral and peripheral membrane proteins associated with the bilayer system contribute approximately one-quarter to one-third of the infrared signal determined for the coated membrane assemblies. The difference spectrum for the clathrin coat that is determined by subtracting the spectrum of the uncoated vesicles from that of the coated system is compared to a clathrin spectrum that reflects the lattice network of protein reassembled in the absence of a vesicle membrane. The similarity of the frequency and intensity characteristics of these forms of clathrin emphasizes the nearly identical secondary structures comprising the two separate assemblies. Thus, the water-soluble, reconstituted cage-type structures should provide a convenient model system for membrane-bound clathrin in spectroscopic investigations involving clathrin–membrane interactions. Studies specifically concerning the properties of reassembled cages under defined conditions will be reported at another time.

The close correspondence of the spectra of the integral and peripheral membrane protein components present in uncoated vesicles to the spectra recorded for the reassembled clathrin cages suggests the existence of very similar secondary structures within the quite different aqueous and hydrophobic environments. Further resolution of this point must await the determination of the protein structures associated with the vesicle bilayer. Finally, we note that the amide I and II spectral regions for the clathrin triskelions differ significantly from the spectra determined for the reconstituted cages. Both the observed frequency parameters and the amide II/amide I intensity ratios indicate a decreased α-helical content for the polypeptide chains as the water-soluble triskelion components assemble into the cagelike lattice forms characteristic of coated vesicles.

Acknowledgments

We thank Dr. Ralph G. Adams for his aid in obtaining spectra and for numerous helpful discussions. We also express our thanks to W. H. Jennings and J. Powell for their expert computer assistance.

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Absence in Papaya Peptidase A Catalyzed Hydrolyses of a pK_a ~ 4 Present in Papain-Catalyzed Hydrolyses†

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ABSTRACT: The pH dependence below pH 6 of k_{cat}/K_m for the papaya peptidase A catalyzed and papain-catalyzed hydrolyses of the *p*-nitrophenyl esters of hippuric acid, benzyloxycarbonylglycine, and benzyloxycarbonyl-L-lysine has been determined and compared. In the case of papain, k_{cat}/K_m was observed to be modulated by two acidic ionizations, whereas for papaya peptidase A a single ionization controls the pH dependence of k_{cat}/K_m below pH 6. A comparison of the values of the pH-independent parameter k_{cat}'/K_m' for the two enzymes toward the three substrates indicates that papaya peptidase A performs catalytically as well as papain without reflecting the specificity requirements known to be essential for the catalytic efficiency of the latter enzyme.

For the hydrolysis of specific substrates catalyzed by papain (EC 3.4.22.2), the pH dependence of k_{cat}/K_m is generally observed to be bell shaped with pK_a values of about 4 and 8.5 (Glazer & Smith, 1971; Lowe, 1976). Originally, these pK_a values were ascribed to the ionizations of, respectively, a carboxylic acid group and the thiol group of the active-site cysteine (Smith & Parker, 1958). However, with the seeming acceptance of the thiolate–imidazolium ion pair formed between cysteine-25 and histidine-159 as the predominant and catalytic competent enzyme form in the pH range 4–8.5 [Drenth et al., 1975; Polgar, 1977; Brocklehurst et al. (1981) and references cited therein], a new view on the catalytic mechanism of papain and in particular on the assignment of the observed pK_a values has been established.

During the past decade evidence has accumulated for the influence of a second acidic ionizing group on the active-center characteristics and catalytic activity of papain (Brocklehurst & Little, 1970; Sluyterman & Wijdenes, 1973; Bendall & Lowe, 1976a,b; Lewis et al., 1978). Thus, the formerly controversial assignment of the pK_a of about 4 to either histidine-159 or aspartic acid-158 now seems replaced with the contention that two deprotonations resulting in the formation of the S^-–ImH^+ interactive system and the carboxylic anion of aspartic acid-159, respectively, are reflected in the acidic limb of the pH profile for papain-catalyzed hydrolyses.

In the initial investigations of papaya peptidase A, that enzyme was shown to catalyze the hydrolysis of casein and urea-denatured hemoglobin with pH dependences quite similar to those observed for papain (Schack, 1967), and the (k_{cat}/K_m)–pH profile for the papaya peptidase A catalyzed hydrolysis of *N*-benzoyl-L-arginine ethyl ester was shown to be bell shaped with apparent pK_a values of 4.3 and 8.5 (Robinson, 1975). However, recent studies of the pH dependence of the rate of irreversible inhibition by 2,2′-dipyridyl disulfide of the three monothiol proteases ficin (EC 3.4.22.3), actinidin, and papaya peptidase A have revealed that these enzymes appear to lack an ionizing group with the characteristics attributed to the aspartic acid-158 residue of papain (Malthouse & Brocklehurst, 1976; Brocklehurst et al., 1981; Baines & Brocklehurst, 1982). In order to establish whether this observation extends to the catalytic action of papaya peptidase A, we have carried out a comparative study of the acidic limbs of the (k_{cat}/K_m)–pH profiles for papain-catalyzed and papaya peptidase A catalyzed hydrolyses of specific substrates. The results presented in this work show that below pH 6 the pH dependence of k_{cat}/K_m is determined by two ionizations for papain-catalyzed hydrolyses but by only a single ionization for papaya peptidase A catalyzed hydrolyses.

Materials and Methods

Enzymes. Dried papaya latex (crude, type 1) was obtained from Sigma Chemical Co. Spray-dried papaya latex was generously supplied by Powell & Scholefield Ltd., Liverpool, U.K. From these materials, papain and papaya peptidase A were purified to the level of 1.0 mol of SH/mol of protein by procedures described elsewhere (Kaarsholm & Schack, 1983; P. Schack and N. C. Kaarsholm, unpublished results). The enzymes were stored at 4 °C as the mercury derivative until required.

Substrates. *N*-Benzyloxycarbonylglyine *p*-nitrophenyl ester (Z-Gly-ONp)[1] was obtained from Sigma Chemical Co., and *N*-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester (Z-Lys-

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[1] Abbreviations: pHNP, hippuric acid *p*-nitrophenyl ester; Z-Gly-ONp, benzyloxycarbonylglycine *p*-nitrophenyl ester; Z-Lys-ONp, benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

0006-2960/84/0423-0631$01.50/0