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Further Studies with Isolated Absolute IR Spectra of Bacteriorhodopsin Photocycle Intermediates: Conformational changes and possible role of a new proton-binding center

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Abstract

We recently published procedures describing the isolation of absolute IR spectra for all of the intermediates of the bacteriorhodopsin photocycle, and from these to obtain transitional difference spectra between consecutive intermediates (Hendler et al. *Applied Spectroscopy*, 65, 1029-1045 (2011)).

In that work, we concentrated mainly on proton-binding centers and the route of proton transport across the membrane. In the current communication we use isolated spectra for the Amide I, Amide II, and Amide III envelopes to obtain quantitative information on the extents of conformational change accompanying each transition in the photocycle. Our main finding is that most of the conformational changes occur in the conversion of the M_F intermediate to N.

In the earlier publication, a new proton acceptor, absorbing at 1650 cm⁻¹ was identified which appeared to accept a proton from Asp96COOH during the transformation of [†]BR* to L. Below, we present evidence which supports this interpretation and propose a possible role for this new component.

Introduction

We recently described new approaches that enabled us to isolate individual absolute and difference spectra for all of the bacteriorhodopsin photocycle intermediates.¹ In interpreting these results, we relied heavily on a large number of previously published FTIR studies (see

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[†](BR*): photon-activated BR (authentic spectrum not isolated here); BR: ground state of photocycle ; bR: the protein.

ref. 1 for these citations). In the current study attention is focused on the isolated spectra for the Amide I, II, and III regions.

Protein conformational changes are reflected in particular vibrational modalities of the peptide bond that are seen in three regions of the IR spectrum. The strongest of these occur in the Amide I region from 1700 cm^{-1} to 1600 cm^{-1} , and emanate from C=O stretching weakly coupled with C-N stretching, and N-H in-plane bending. The next strongest signals occur in the Amide II region from 1600 cm^{-1} to 1500 cm^{-1} , and reflect N-H in-plane bending weakly coupled with C-N stretching. A much weaker set of signals is seen in the Amide III region from 1350 cm^{-1} to 1200 cm^{-1} . These reflect N-H in-plane bending coupled with C-N stretching and also include C-H and N-H deformation modes. Because of their strong signals, the Amide I and II regions have received the most attention in attempts to quantify conformational changes. The Amide III region has been largely ignored because of the weak signals it presents. However, there is a wealth of information that can be gathered from changes seen to occur in that region. Using our cleanly isolated absolute spectra, we examined all three regions. The one consistent finding is that the maximum extents of conformational changes occur during the transition between the M_F and N intermediates in the M_F path of the bacteriorhodopsin photocycle (discussed below). In addition, significant structural differences between the M_F and M_S intermediates were seen in all three spectral regions.

With the combined spectra of both M_F and M_S (as is normally done), we confirmed the consensus view on the proton path.¹ Using separate spectra for the M_F and M_S intermediates, we were able to distinguish important differences between the functions of the two separate pathways for M_F and M_S decays as defined in the parallel cycles model of Hendler et al.¹⁻³

The subscripts F and S refer to the faster and slower decay time constants (τ) for the intermediates. The asterisks indicate that only the photo-activated forms of bacteriorhodopsin undergo photocycles. Our time resolution does not allow the isolation of these states. The spectra we obtain are for the ground states. It was shown that protons traveling the M_F path form a pH , while protons traveling the M_S path build a membrane potential (ψ).¹ In the current communication, attention is focused mainly on conformational changes that accompany each transition in the photocycle.

Although, most thoroughly studied, the assignment of vibrational frequencies in the Amide I region to specific protein or peptide configurations is not an exact science. However, in the case of soluble proteins, some real progress has been made.⁴⁻⁷ In some instances, Amide I band frequency assignments for particular types of secondary structure, obtained from soluble proteins have been found to apply to membrane proteins. In ref. 5, equations were presented, which evaluate relative amounts of α -helix, β -sheet, β -turn and random coil, based on absorbances at a series of specified frequency ranges. In a review, Goormaghtigh, et al. defined specific regions where different secondary structures tend to absorb.⁷ However;

progress in the case of membrane proteins has lagged far behind what has been reported for soluble proteins. In the first part of the studies reported below, we have examined the Amide I, Amide II, and Amide III regions for the extents of conformational changes.

The X-ray crystallographic structure of bR at 1.55 Å resolution shows that 171 amino acid residues (69%) are in α -helices, 67 (27%) in loops and turns, and 10 (4%) form a twisted anti-parallel β -sheet on the external surface connecting helices B and C.⁸ Since the predominant stable structure is α -helix, we assume that most of the changes result from changes in tilts and immediate environments rather than major changes in structure *per se*. Our main purpose is to quantify extents of conformational changes rather than to identify specific changes in protein structure.

In the earlier publication¹, a previously unrecognized proton-binding center (X) with a characteristic frequency at 1650 cm⁻¹ was described. In the second part of the work presented here, we suggest a possible role for X.

Methods

All procedures for obtaining the isolated absolute and transitional difference spectra used here were previously described in detail.¹ The spectra are averages of 2200 repeats using a Step Scan protocol and fitting procedures for the kinetic constants which presented very low errors and dependencies. In this work, to obtain quantitative data on the extents of conformational change based on the isolated absolute IR spectra for each intermediate, we developed some new approaches.

1. The absolute IR spectra for pairs of consecutive photocycle intermediates contained within the frequency ranges of the Amide I, Amide II, and Amide III envelopes were compared to reveal directly the extents of differences.
2. Difference spectra between these pairs of intermediates were examined to determine all of the distinct peaks and troughs commonly seen throughout the photocycle. In this way, we identified separate components in the envelope profiles. Using the characteristic frequencies for these components, we quantified the extents of change in these components for each successive transition in the two decay paths, by averaging the $\pm$ A at each central wave number with its two adjacent neighbors, and plotted these against the corresponding steps in the photocycle. The difference spectrum between M_F and M_S intermediates was also included in these analyses.

Results

A. Extents of conformational changes in the Amide I envelope between consecutive intermediates in the BR photocycle

Figures 1 and 2 show absolute IR spectra for pairs of consecutive intermediates contained within the Amide I envelope. It is apparent that the most extensive changes occur in the M_F → N transition (Fig. 1C) and the least between BR and the L intermediates (Fig. 1A).

Figure 2 shows pairs of intermediates for the $L \rightarrow M_S$ (Fig 2A) and $M_S \rightarrow BR$ (Fig 2B) transitions in the M_S path of the photocycle. It is clear that the extents of conformational changes are much less in the M_S than the M_F pathway. In addition, we show in Fig 2C, conformational differences between M_F and M_S .

Quantification based on amplitudes in difference spectra

In order to assess the number of individual components contributing to the photocycle changes seen in the Amide I envelope, we looked at the difference spectra for each transition depicted in Figs. 1 and 2. These difference spectra displayed distinct peaks and troughs and are presented in the Supplementary Material (Figs S1 for the M_F path and Fig. S2 for the M_S path). This analysis produced 11 characteristic peaks and troughs as likely indicators of conformational changes. These are located at frequencies (cm^{-1}) 1696, 1689, 1684, 1674, 1667, 1653, 1643, 1637, 1630, 1625, and 1602.

To quantify the accumulative extents of change, referenced to the ground state BR, in these 11 features for each successive transition in the two decay paths, we averaged the $\pm A$ at each central wave number with its two adjacent neighbors. The results for the M_F path are shown graphically in Fig. 3 and quantitatively in Table I. Corresponding data for the M_S path are shown in Fig. 4 and Table II.

Figure 4, using the same scaling as in Fig. 3, shows that the extents of conformational changes were far less than in the M_S path than seen in the M_F path. The extents of structural differences between M_F and M_S are listed in Table III.

B. Extents of conformational changes in the Amide II envelope between consecutive intermediates in the BR photocycle

Figure 5 shows absolute IR spectra for pairs of consecutive intermediates contained within the Amide II envelope in the M_F path. It is apparent that the most extensive changes occur in the $M_F \rightarrow N$ transition (Fig. 5C) and the $O \rightarrow BR$ transition (Fig. 5E). Figure 6 shows the same information for steps in the M_S path and the differences between M_F and M_S .

The difference spectra between consecutive intermediates in the Amide II envelope for both the M_F and M_S paths are shown in the Supplementary material (Figs S3 and S4). Seven peaks and troughs were seen. One of these at 1557 cm^{-1} is for uncharged Arg82 and another for $^{\ddagger}\text{C}=\text{C-SBH}$ is at 1525 cm^{-1} . The other five shared in more than one panel were located at frequencies (cm^{-1}) 1585, 1545, 1534, 1515, and 1508. These changes quantified as described above for Amide I are shown graphically in Fig. 7 and quantitatively in Table IV. Corresponding data for the M_S path are shown in Fig. 8 and Table V. Shading in the Tables indicates the maximum change at each wave number.

It is important to note that the large amplitude seen at 1557 cm^{-1} , which coincides with the frequency for uncharged Arg82, is not associated with the deprotonation of Arg82⁺. Instead, as concluded in ref 1, it more likely signals conformational and/or environmental changes.

[‡]C=C-SBH: protonated Schiff base

Cumulative changes in amplitudes (mOD) at each characteristic wave number relative to BR in the M_S path are shown in Table V. The midpoint was averaged with its two neighbors. Because there is no clear consensus on a common intermediate showing a consistent maximum change, shading is not used in Table V. As was the case for Amide I, the changes in the M_S path, were much smaller than seen in the M_F path. Table VI shows the differences between M_F and M_S in the Amide II envelope.

C. Extents of conformational changes in the Amide III envelope between consecutive intermediates in the BR photocycle

Figure 9 shows absolute IR spectra for pairs of consecutive intermediates contained within the Amide III envelope in the M_F path. Significant changes are seen to accompany each transition. Figure 10 shows the same information for steps in the M_S path and the differences between M_F and M_S .

Difference spectra between consecutive intermediates in the Amide III envelope for both the M_F and M_S paths are shown in Supplementary material (Figs. S5 and S6). There are nine commonly shared peaks and troughs located at frequencies (cm^{-1}) 1336, 1329, 1320, 1312, 1300, 1270, 1254, 1232, and 1208. Changes in amplitude, quantified as described above for Amide I, are shown graphically in Fig. 11 and quantitatively in Table VII for the M_F path. Corresponding data for the M_S path are shown in Fig. 12 and Table VIII. Shading in the Tables indicates maximum change at each wavenumber.

The significance of the Amide III region has been most thoroughly studied by B. R. Singh and associates, who use model proteins with secondary structures determined by X-ray crystallography and a variety of deconvolution and analytical methods to determine the number of components contained within the envelope.⁹⁻¹¹ The yellow, cyan, and green backgrounds in Table VII denote wave number ranges for α -helix, turn, and β -sheet conformations according to Singh and co-workers. Although, none of the model calibration standards was a membrane protein, the appearance of our spectra and number of features are quite similar.

D. Kinetics and possible role for newly identified proton-binding group (XH) with a frequency at 1650 cm^{-1}

Figure 13 (panel A) compares the time course for Asp96COOH at 1740 cm^{-1} (blue) with that of XH 1650 cm^{-1} (green). Panel B compares the time course of XH at 1650 cm^{-1} (blue) with that of C=C-SBH at 1524 cm^{-1} (green). Panel C shows corresponding time courses for all of the intermediates.

Discussion

In a recent comprehensive review on conformational changes in the bR photocycle, from findings based on electron and X-ray crystallographic analyses, Hirai and Subramiam reviewed a large number of publications (cited therein) from many laboratories.¹² The main conclusion was that no consensus had been reached. Specifically, they found the structures for the same intermediate reported by multiple research groups are not only significantly

different, but are also not on the same trajectory of conformational change of the protein. We are not surprised by this analysis and conclusion for many reasons.

1. Because of the overlap of all intermediates at any point in time of the photocycle there is always a mixture of several intermediates (see Fig. 13C above).
2. Trapping procedures based on rapid freezing, and the use of mutants, or varying conditions such as pH or temperatures result in different photocycles where different, unquantified degrees of contamination are present.
3. Hydrophobic binding is considerably weakened at low temperatures.

There is no way to ensure that any of these procedures can lead to isolated intermediates relevant to any one kind of photocycle, nor that studies pursued in any particular laboratory should produce the same results as obtained in any other laboratory. Even if it were possible to reproducibly obtain unique structures for the intermediates by the methods used, what is the relevance of structures obtained and analyzed as described above to the real photocycle that occurs in a fully hydrated membrane at ambient temperatures?

As reported here, new approaches, based on our recent isolation of IR absolute spectra for all of the intermediates¹, revealed that the extents of change are most pronounced during the $M_F \rightarrow N$ transition and reversed during the $N \rightarrow O \rightarrow BR$ transitions. What needs to be done next is to define precisely the specific conformational changes that have occurred and how they relate to the formation of the electrochemical potential that drives ATP synthesis. To this end, we have been developing instrumentation and procedures for obtaining isolated X-ray diffraction maps for each intermediate, using the same mathematical deconvolution methods described in this and the preceding publication.¹ An early indication of our present findings was that IR markers in the Amide I and Amide II regions steadily increased from the ground state (BR) to the N intermediate.¹³ Using discrete sampling techniques to minimize cross contamination of overlapping peaks, Lórenz-Fonfría et al. reported that most of the structural changes during the photocycle accompany the formation of the N intermediate.¹⁴ We also found evidence of significant structural differences between M_F and M_S . In this respect, we confirm the electron crystallographic findings of Subramaniam et al.¹⁵

Possible role for the newly discovered proton-binding and release component X at 1650 cm^{-1}

We have recently reported, using isolated absolute spectra, that a proton lost by Asp96COOH in the $BR \rightarrow L$ transformation is bound by a component whose protonated form absorbs at 1650 cm^{-1} . It was also found that in the $L \rightarrow M_F$ transition, the proton is transferred back to Asp96COO⁻. The time profiles in Fig. 13 (above) document this sequence of events. These findings raise some important questions.

1. What is the identity of X?
2. What is its function?
3. Why is a proton transferred from Asp96 to X in the $BR \rightarrow L$ transformation returned back to Asp96 in the $L \rightarrow M_F$ transition?

Questions 2 and 3 are related. We do not believe, and do not propose, that X should be inserted into the proton flow pathway that has been established, and which our work confirms, when we use the combined form of M_F and M_S . The parallel cycles model which led to the isolated absolute IR spectra, presents two separate pathways for the decays of M_F and M_S back to their respective ground states. One additional question which we have not addressed is how the M_S pathway obtains a proton from the external cytoplasmic surface? In the pathway using either undivided M or in the M_F pathway, the external proton is acquired by Asp96COO^- via an aqueous channel during the $N \rightarrow O$ transition. However, the N and O intermediates are not present in the M_S pathway.

The model we present below is one that offers a possible answer to all of the questions posed above. At this point, the explanation is only a supported speculation to be further evaluated by future work.

In the M_F pathway, a cytoplasmic proton has access to Asp96 in the $N \rightarrow O$ transition, and then is subsequently transferred to SB in the $M_F \rightarrow N$ transition, but, neither of these transitions is present in the M_S pathway. If, the only available route for supplying an external proton to the photocycle is in the $N \rightarrow O$ transition, then this proton must be funneled to the M_S pathway, as needed. With X in contact both with Asp96 and SB, the scheme below can operate.

There are still the same two separate pathways, and X serves as a channel and proton buffer for both. The function of X could be as a conduit and regulator for the distribution of protons between the two pathways as needed for the buildup of pH and ψ .¹ As to the identity of X, its characteristic frequency at 1650 cm^{-1} suggests that it could be one of the other arginines (not Arg82) present in bR.

On the differences between the isolated M_F and M_S spectra

In our previous paper based on isolated absolute IR spectra of the intermediates, we noted a difference in proton distribution between Asp85 and SB in the M_F and M_S intermediates (Fig. 8 of ref. 1), and stated that we would present an interpretation after further consideration, which we now present.

The M intermediate is formed in the $L \rightarrow M$ transition where a proton is transferred from $\text{C}=\text{C-SBH}^\ddagger$ to Asp85. Previous analyses of the photocycle were based only the combined M spectrum. If the proton is completely transferred, there should be no $\text{C}=\text{C-SBH}$ in M. In the parallel cycles model, there are two separate ground states, two separate L's and two

[‡] $\text{C}=\text{C-SBH}$: protonated Schiff base

separate M's. Since there is still only one proton transferred per bR molecule, a statistical fraction goes through each path. Previously, it was shown that the two forms of M are functionally different¹, and in the present communication they are seen to differ in structure. Therefore, the pK's of Asp85COOH and C=C-SBH in M_F and M_S need not be identical. The distribution of H⁺ between Asp85 and SB is determined by the pK's for SB in M_F and M_S *vis-à-vis* the pK for Asp85. The earlier publication showed that Asp85 becomes more protonated in the L → M_F transition than in the L → M_S transition (compare Fig. 11A to 11D).¹ More protonated Asp85 means less protonated SB. This suggests that the M_S formed has less Asp85COOH and more C=C-SBH than M_F. The higher content of Asp85COOH in M_F than M_S is clearly shown by the peak at 1763 cm⁻¹ in the M_F – M_S difference spectrum (Fig. 8 of ref. 1). This is consistent with and can explain the trough at 1524 cm⁻¹ in the M_F – M_S difference spectrum.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Absolute spectra of consecutive intermediates in M_F path (Amide I).

Superimposition of absolute spectra in the Amide I region for consecutive intermediates in the M_F path.

Figure 2.

Absolute spectra of consecutive intermediates in M_S path and comparison of M_F and M_S (Amide I).

Superimposition of absolute spectra in the Amide I region for consecutive intermediates in the M_S path (panels A and B). Although not directly linked in the photocycle, conformational differences between M_F and M_S are shown in panel C.

Figure 3.

Changes in mOD at 11 frequencies in M_F path (Amide I).

The six points in the abscissa are for the sequential intermediates BR, L, M_F , N, O, and BR in the M_F pathway. The eleven graphs show the quantified changes at each characteristic frequency. The vertical green lines are drawn at intermediate N and the plotted $\pm A$ amplitudes as listed in Table I.

Figure 4.

Changes in mOD at 11 frequencies in M_S path (Amide I).

The four points in the abscissa are for the sequential intermediates BR, L, M_S , and BR in the M_S pathway. The eleven numbered graphs correspond to the numbered rows and corresponding wave numbers listed in Table II.

Figure 5. Absolute spectra of consecutive intermediates in M_F path (Amide II). Superimposition of absolute spectra in the Amide II region for consecutive intermediates in the M_F path.

Figure 6.

Absolute spectra of consecutive intermediates in M_S path and comparison of M_F and M_S (Amide II).

Superimposition of absolute spectra in the Amide II region for consecutive intermediates in the M_S path. Although not directly linked in the photocycle, conformational differences between M_F and M_S are shown in panel C.

Figure 7.

Changes in mOD at 7 frequencies in M_F path (Amide II).

The six points in the abscissa are for the sequential intermediates BR, L, M_F , N, O, and BR in the M_F pathway for the characteristic frequencies listed above. The vertical green lines are drawn at intermediate N and the plotted amplitudes are listed in Table IV. The asterisks for frequencies 1557 cm^{-1} and 1525 cm^{-1} refer to the fact that the scales for the former (Arg82 (neutral)) and the latter (protonated Schiff base) are 2.5 greater than those at the other frequencies. This is also shown by the relative heights of the vertical green bars drawn at the N intermediate and which represent 80 mOD of change

Figure 8.

Changes in mOD at 7 frequencies in M_S path (Amide II).

The four points in the abscissa are for the sequential intermediates BR, L, M_S , and BR in the M_S pathway. The seven numbered graphs correspond to the numbered rows and corresponding wavenumbers listed in Table V.

Figure 9.

Absolute spectra of consecutive intermediates in M_F path (Amide III).

Superimposition of absolute spectra in the Amide III region for consecutive intermediates in the M_F path.

Figure 10.

Absolute spectra of consecutive intermediates in M_S path and comparison of M_F and M_S (Amide III).

Superimposition of absolute spectra in the Amide III region for consecutive intermediates in the M_S path. Although not directly linked in the photocycle, conformational differences between M_F and M_S are shown in panel C.

Figure 11.

Changes in mOD at 9 frequencies in M_F path (Amide III).

The six points in the abscissa are for the sequential intermediates BR, L, M_F , N, O, and BR in the M_F pathway. The nine numbered graphs correspond to the numbered rows and corresponding wave numbers listed in Table VII. The vertical green lines are drawn at intermediate N and the plotted $\pm$ amplitudes are listed in Table VII.

Figure 12.

Changes in mOD at 9 frequencies in M_S path (Amide III).

The four points in the abscissa are for the sequential intermediates BR, L, M_S , and BR in the M_S pathway. Scaling is the same as in Fig. 11. The nine numbered graphs correspond to the numbered rows and corresponding wave numbers listed in Table VIII. Differences between the M_F and M_S intermediates are shown in Table IX.

Figure 13.

Relationship of protonations and deprotonations among AspCOOH, SBH, and X at 1650 cm⁻¹ to each other and to the time course profiles of the photocycle intermediates.

The small magnitudes for changes of absorbance in panels A and B were scaled to match each other and, the absorbance values shown in panel C. For the green trace in panel A, a gain of 250 and an offset of 0.22 were used; for the blue trace, a gain of 100 and an offset of 0.9 were used. For the blue trace in panel B, a gain of 40 and an offset of 0 were used; for the green trace, a gain of 20 and an offset of 0.2 were used.

The vertical blue and green lines in panel A show that the time courses for Asp96COOH and XH are mirror images during the BR → L → M_F stages. This finding corresponds to difference spectra which show that Asp96COOH is deprotonated during the BR → L transformation and reprotonated during the L → M_F transition.¹ The reprotonation of Asp96COO⁻ occurs during the N → O transition. Panel B shows a donor-acceptor relationship between SB and X during the conversion of BR to L.

Table I

Amide I cumulative changes in amplitudes (mOD) at each intermediate relative to BR in the M_F path

wave number (cm ⁻¹)	BR	L	M _F	N	O	BR
1696	0	-4.1934	-11.3612	-33.2623	-17.9382	0
1689	0	-3.3955	-7.4821	-15.9572	-11.6790	0
1684	0	-2.4939	-5.3947	-11.1565	-5.5868	0
1674	0	-3.1139	-15.0086	-55.8166	-24.1135	0
1667	0	-3.3735	-9.6661	-54.3496	-25.6135	0
1653	0	2.5339	2.3903	21.9138	8.1056	0
1643	0	3.1271	-10.6883	-34.7582	-9.3163	0
1637	0	-1.9352	-11.9717	-20.4228	-8.7701	0
1630	0	5.4881	-2.5133	0.7591	5.1915	0
1625	0	9.2321	1.9501	-7.7191	8.3165	0
1602	0	9.2321	-5.2057	-19.8785	-9.1275	0

Note: The maximum changes (shaded) occurred mostly at the N intermediate.

Table II

Amide I cumulative changes in amplitudes (mOD) at each intermediate relative to BR in the M_S path.

wave number (cm ⁻¹)	BR	L	M _S	BR
1696	0	-4.1934	0.6982	00
1689	0	-3.3955	-3.9117	0
1684	0	-2.4939	-1.7770	0
1674	0	-3.1139	6.6154	0
1667	0	-3.3735	2.8778	0
1653	0	2.5339	-13.9023	0
1643	0	3.1271	-0.8560	0
1637	0	-1.9352	8.0422	0
1630	0	5.4881	0.3470	0
1625	0	9.2321	8.6072	0
1602	0	2.0052	2.7006	0

Note: The maximum changes in amplitudes are shaded.

Table IIIAmide I $M_F - M_S$ differences

wave number (cm^{-1})	delta A (mOD)
1696	12.06
1689	3.57
1684	3.62
1674	21.62
1667	12.54
1653	-16.29
1643	9.83
1637	3.93
1630	2.86
1625	6.66
1602	7.91

Table IV

Amide II cumulative changes in amplitudes (mOD) at each intermediate relative to BR in the MF path

Wave number (cm ⁻¹)	BR	L	M _F	N	O	BR
1585	0	-0.7348	-5.7134	-6.3741	-4.6484	0
1557	0	-0.6867	22.1833	81.5068	47.0981	0
1545	0	3.0732	-10.4140	-0.4648	-0.0451	0
1534	0	-10.0511	-30.1238	-6.4381	-11.5702	0
1525	0	-27.9144	-55.1132	-61.5929	-46.1896	0
1515	0	-5.4534	-20.4997	-34.5622	-17.2947	0
1508	0	0.7532	-4.0836	-6.1354	-12.0956	0

Note: The maximum changes (shaded) occurred mostly at the N intermediate.

Table V

Amide II cumulative changes in amplitudes (mOD) at each intermediate relative to BR in the M_S path.

wave number (cm ⁻¹)	BR	L	M _S	BR
1585	0	-0.7348	-0.4710	0
1557	0	-0.6867	2.9445	0
1545	0	3.0732	-0.4457	0
1534	0	-10.0511	-17.1943	0
1525	0	-27.9144	-27.8482	0
1515	0	-5.4534	-6.9755	0
1508	0	0.7532	0.4987	0

Table VIAmide II $M_F - M_S$ differences

wave number (cm^{-1})	delta A (mOD)
1585	5.24
1557	-19.24
1545	9.97
1534	12.93
1525	27.26
1515	13.52
1508	4.58

Table VII

Amide III mOD Changes in amplitudes at each intermediate relative to BR

Wave number (cm ⁻¹)	BR	L	M _F	N	O	BR
1336	0	0.0451	-1.8919	-1.4738	0.0828	0
1329	0	-0.1426	-0.1170	7.0868	1.6295	0
1320	0	-2.1854	-3.7652	-5.6338	-1.7168	0
1312	0	-1.2084	-3.9793	1.4597	-0.9368	0
1300	0	1.5113	0.1479	11.1025	4.5911	0
1270	0	-3.1045	-2.3798	-9.0624	-2.9957	0
1254	0	-8.3418	-14.7381	-26.2879	-15.8792	0
1232	0	0.4982	0.1410	2.7482	0.6056	0
1208	0	-7.1067	-9.6221	-7.9633	-6.1417	0

Table VIII

Amide III mOD Changes in amplitudes at each intermediate relative to BR

wave number (cm ⁻¹)	BR	L	M _S	BR
1336	0	0.0451	1.0093	0
1329	0	-0.1426	-1.8207	0
1320	0	-2.1854	-2.0732	0
1312	0	-1.2084	-1.0505	0
1300	0	1.5113	-0.8791	0
1270	0	-3.1045	-1.0249	0
1254	0	-8.3418	-9.5446	0
1232	0	0.4982	-2.9973	0
1208	0	-7.1067	-8.7973	0

Table IX

shows the differences between M_F and M_S in the Amide III envelope.

Table IX Amide III $M_F - M_S$ differences	
wave number (cm^{-1})	delta A (mOD)
1336	2.90
1329	-1.70
1320	1.69
1312	2.93
1300	-1.03
1270	1.35
1254	5.19
1232	-3.14
1208	0.82