Secondary Structural Analysis of Two Recombinant Murine Proteins, Interleukins 1α and 1β: Is Infrared Spectroscopy Sufficient To Assign Structure?

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ABSTRACT: The secondary structure for two murine recombinant proteins, interleukins 1α and 1β (rmIL-1α and -1β), has been analyzed by Fourier transform infrared (IR) spectroscopy and then compared to results obtained by X-ray diffraction, circular dichroism (CD), and nuclear magnetic resonance (NMR) spectroscopy. The IR results obtained here for rmIL-1α and -1β suggested that their secondary structures consisted predominantly of β-sheets or strands. However, the analysis also revealed a significant absorption band near 1656 cm^{-1}, which is typically assigned to α-helical or random structures. When these same murine polypeptides were analyzed by CD, no evidence of α-helical structures was observed. Further, published X-ray diffraction and NMR studies characterizing the human forms of IL-1α and -1β indicate the absence of α-helices and that the human proteins are composed mainly of β-strands (i.e., >55%), with approximately 24% of the amino acids involved in large loops connecting the strands. The murine IL-1 proteins, when compared to their respective human counterparts, each show greater than 80% sequence homology. Given this fact, the CD analyses, and the result that this IR band amounted to 21% of the overall integrated area, the absorption peak at 1656 cm^{-1} was attributed to the presence of large loops rather than to α-helical or random structures. Such a structural assignment appears reasonable and is totally consistent with the established existence of large loops in the human forms as well as in other proteins found to fold similarly (viz., human bFGF). More importantly, these findings indicate that IR alone is not sufficient to unequivocally establish secondary protein structure without verification by other analytical methods and suggest that investigators should exercise caution in the assignment of IR bands.

The growing number of proteins analyzed by techniques like X-ray diffraction, nuclear magnetic resonance (NMR), circular dichroism (CD), and Fourier transform infrared spectroscopy (IR) over the last decade has significantly improved and enhanced the ability to understand important correlations between bioactivity and secondary structure. One analytical method which has been steadily evolving and which has resulted in a large number of publications describing secondary protein structure has been IR. This particular method has as its prime advantage the ability to rapidly acquire high signal-to-noise spectra in aqueous solutions, particularly in situations where single crystals (for X-ray) are unavailable or the complex NMR assignments have not been worked out. Under many circumstances, the utility of IR to characterize protein structure is enhanced with before-mentioned techniques like CD, X-ray, or NMR. For instance, CD is quite sensitive to the presence of α-helices but is sometimes unable to definitively identify turns or certain β-structures due to inherently weak signals (Chang et al., 1978). On the other hand, IR is capable of distinguishing numerous types of β-structures and turns. Accordingly, it is possible to coalesce the results obtained from CD and IR in order to achieve a better understanding of secondary structure for a particular protein. Both these techniques suffer from the disadvantages that they do not establish three-dimensional location (like X-ray and NMR) and that structural assignments are made on the basis of theoretical calculations or by comparison with known protein structural data bases. Despite the limited nature of such data bases and theoretical models, the tendency has been to unequivocally assign specific absorption bands to ideal or well-defined types of secondary structural elements (e.g., α-helices, 3_{10} helices, antiparallel β-sheets, type II turns). The problem with such assignments is that secondary protein structures often present a continuum of specific types rather than a strictly defined three-dimensional arrangement for a series of amino acids. In this paper, we have the unique opportunity to analyze the secondary structure of two proteins by IR and CD and then make comparisons with the definitive structural assignments derived from separate X-ray diffraction and NMR studies.

Interleukins 1α and 1β are two polypeptides which have important functions in both primary immune and inflammatory responses, exerting their effects in nearly every tissue or organ throughout the body (Dinarello, 1991). Interestingly, although the IL-1α and IL-1β forms share less than 30% sequence homology and differ in certain physicochemical properties like their isoelectric points or sensitivity to heat (Schmidt, 1984; Saklatvala et al., 1985; Krakauer, 1985), they bind equipotently to the same receptor (Dower et al., 1986a,b; Kilian et al., 1986) and have nearly identical biological activities (Rupp et al., 1986). In addition, the murine IL-1's are approximately 80% homologous with their human recombinant counterparts (Lomedico et al., 1984; Gray et al., 1986).

Recent reports (Clore et al., 1991; Graves et al., 1990; Driscoll et al., 1990; Finzel et al., 1989) with NMR and X-ray techniques indicate that human IL-1α and -1β are composed primarily of β-strands, tight turns, and large loops with no evidence of α-helices. Our objective in this work was to structurally analyze the murine forms of IL-1 with IR and CD spectroscopy and then corroborate the results with those al-

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ready obtained for the corresponding homologous human proteins by X-ray diffraction and NMR. Overall, such an approach should provide insight into the utility of IR for protein structural determinations.

Experimental Procedures

Interleukins. Recombinant murine interleukins (rmIL-1α and rmIL-1β) were obtained from expression in *Escherichia coli* of a synthetic gene encoding the C-terminal 156 amino acids (residues 115–270) of the murine IL-1α precursor sequence. The protein was isolated from inclusion bodies and purified to homogeneity (Daumy et al., 1987). Murine IL-1β was obtained from expression of a semisynthetic gene in *E. coli*, and the protein (residues 118–269 of the rmIL-1β precursor) was purified to homogeneity from the soluble cytoplasmic fraction (Daumy et al., 1991).

Sample Preparation. For the IR studies, the proteins in 50 mM ammonium formate buffer, pH 6.5, were lyophilized and suspended in 50 mM Tris buffer, pH 7.3, made in D_2O (Cambridge Isotope Laboratories, Inc., 99.9% isotopic purity) to a final concentration of 5% (w/v). The apparent pD of these solutions was adjusted between the range of 6 and 7 with NaOD. To determine the final pD, a factor of 0.4 was added to the reading obtained from the glass electrode (Covington et al., 1968). The solutions were allowed to equilibrate for at least 24 h prior to spectral measurement to ensure complete NH–ND exchange, which was confirmed by the shift of the amide II band to a lower frequency. No loss of biological activity was observed after sample preparation and spectroscopic examination.

Spectroscopy. Infrared transmission spectra of the aqueous protein solutions were measured using CaF_2 windows separated by 6-μm spacers in a demountable liquid cell on a Nicolet 740 FT-IR instrument equipped with a liquid N_2 cooled MCT detector. Typically, 2000 interferograms were signal averaged at an optical retardation of 0.5 cm, apodized with a Happ–Genzel function, and Fourier transformed to yield spectra with a final resolution of 2 cm^{-1}. Spectra were further processed to eliminate water vapor bands. Factors for water vapor subtraction were determined by subtracting a second derivative spectrum of water vapor from the second derivative spectrum of the sample until the region from 1700 to 1800 cm^{-1} was featureless. The effectiveness of the subtraction was judged by examining the second derivative of the subtracted spectrum for bands at 1684, 1670, 1662, 1653, 1646, and 1617 cm^{-1}, which are characteristic for water vapor (Dong et al., 1990).

Once a properly subtracted spectrum was obtained, the bands were narrowed by Fourier self-deconvolution in accordance with the method of Kauppinen et al. (1981). The resolution enhancement factor K was chosen so as to fall well within the signal-to-noise (S/N) limit ascribed to it by the equation $K < \log (S/N)$. To determine the S/N ratio, the spectra were examined in the region of 1700–1740 cm^{-1}, which was found to be free of any interfering bands. Routinely, S/N ratios greater than 900 up to 1200 were obtained for all spectra. The final bandwidth (HWHH) was chosen by exploring a range of values until consistent bands were produced without significant side lobes or periodic noise. In all cases, the largest possible BW which could satisfy the above conditions was utilized. For both the α and β forms, the optimal band half-width and resolution factors were found to be 14 cm^{-1} and 2.8, respectively; in the deconvolution procedure, a Bessel apodization function and Lorenzian line-shape function were employed. To further confirm the validity of the band selection procedure, second derivative spectra were generated for each sample using the Nicolet SX software. The CD spectra, meanwhile, were obtained with more dilute solutions and subsequently analyzed by established techniques as previously described by Prevelige and Fasman (1987).

Table I: FT-IR Band Frequencies, Fractional Areas, and Proposed Structural Assignments for Murine rmIL-1α

band position (cm^{-1})	band area (%)	proposed assignments
1686	10.9	turns
1673	10.9	turns
1658	21.0	large loops
1649	8.7	CO···water
1637	40.8	β-strands
1625	7.7	β-sheet

Table II: FT-IR Band Frequencies, Fractional Areas, and Proposed Structural Assignments for Murine rmIL-1β

band position (CM^{-1})	band area (%)	proposed assignments
1686	4.7	turns
1674	15.0	turns
1656	20.6	large loops
1644	13.1	CO···water
1635	23.5	β-strands
1622	23.1	β-sheet

Table III: Percentage and Types of Protein Secondary Structure Estimated by CD

protein	helix	β-structure	turn	random
rmIL-1α	0	55	25	20
rmIL-1β	0	60	15	25

Curve-Fitting Analysis. The curve fitting was performed on a personal computer with a program that iteratively adjusted several spectral parameters to obtain the best fit. The number and frequency position of the individual bands were determined from the deconvoluted and second derivative spectra. No bands were utilized in the fitting procedure unless they were clearly indicated by both resolution enhancement methods. The baselines were graphically estimated from the spectra and fixed during the fitting procedure. Once the bands were selected, the frequency and bandwidths were fixed along with a band shape of 50% Gaussian. The program then iteratively varied the peak intensities to minimize the root mean square error. Next, the bandwidths and band shape were individually allowed to vary, and the iteration procedure was repeated until no more significant improvements were achieved in the error term. Finally, to determine how robust the overall fit was, all of the parameters including the frequencies were permitted to simultaneously float after the position of the band around 1650 cm^{-1} was displaced by 10 cm^{-1}, much like the approach of Prestrelski et al. (1991,a,b). The goodness of fit (χ^2) and the standard deviation of the fit were calculated, under these conditions, to be less than 0.002 and 0.0002, respectively. After this iterative process was completed, the band areas of the peaks from 1700 to 1600 cm^{-1} were determined and expressed as a percentage of the total peak area in this range.

Results and Discussion

Figure 1 shows the original, the deconvolved, and the second derivative FT-IR spectra for rmIL-1α. Table I lists the frequencies of all the amide I′ component peaks, their relative areas, and putative assignments of individual bands to secondary structure. Similarly, Figure 2 and Table II give the data for rmIL-1β, while the results from the CD experiments are shown in Table III. These results and the overall rationale for each of the assignments are discussed below.

FT-IR: 1600–1640-cm^{-1} Region. Two separate IR bands were observed in this region for rmIL-1α and -1β with the most

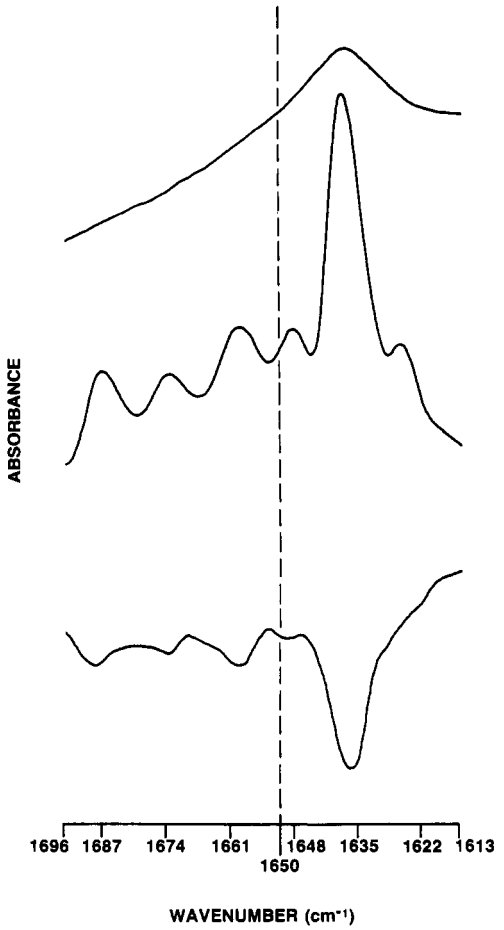

FIGURE 1: Infrared spectra from the amide I′ region for rmIL-1α. The upper, middle, and lower panels depict the original, the deconvolved, and the second derivative spectra, respectively.

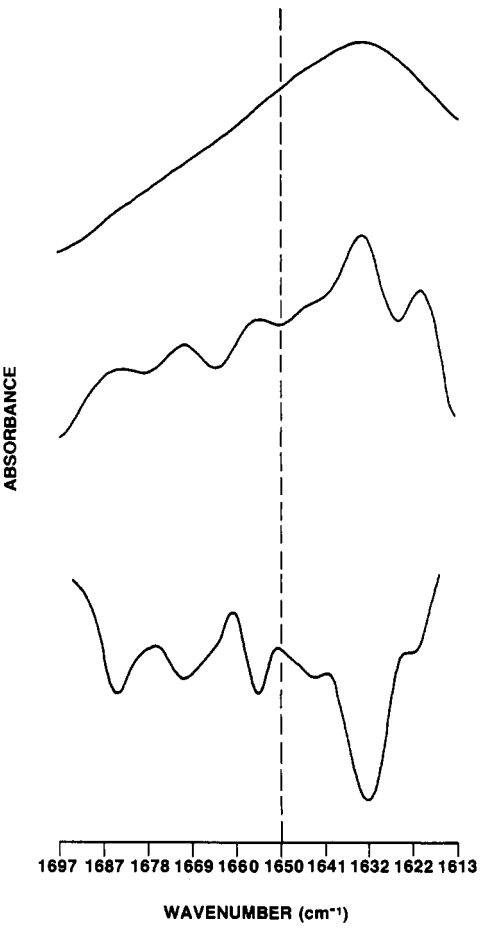

FIGURE 2: Infrared spectra from the amide I′ region for rmIL-1β. The upper, middle, and lower panels depict the original, the deconvolved, and the second derivative spectra, respectively.

prominent peak occurring at approximately 1636 cm^{-1}. The smaller band was positioned at 1622 cm^{-1} for the β form and at 1625 cm^{-1} for IL-1α. Peaks in the upper part of this region (viz., 1620–1645 cm^{-1}) are often assigned to parallel β-sheets (Bandekar & Krimm, 1988) but have also been attributed to antiparallel β-strands (Susi & Byler, 1987). In some instances, protein structures composed of large antiparallel β-sheets would be expected to produce additional bands in the lower part of this region spanning from about 1635 to 1610 cm^{-1} (Krimm & Bandekar, 1986). Thus, the two IR bands in the present study were tentatively assigned to β-strands, probably arranged in an antiparallel configuration. However, it is not possible to uneqivocally distinguish between antiparallel and parallel β-strands using just IR spectroscopy (Susi & Byler, 1987). Interestingly, while the total percentage of these two peaks was nearly the same for both protein forms (i.e., 47% vs 48%), the individual contributions were quite different. For rmIL-1α, the 1637-cm^{-1} band accounts for 41% of the total area compared to only 23% for the β form. This difference indicates that the structural characteristics for the β-strands of the two protein forms were not the same. The existence of a larger band around 1620 cm^{-1} for rmIL-1β may be attributed to the presence of β-structures with a greater extent of intermolecular H-bonding (Wantyghem et al., 1990).

FT-IR: 1660–1700-cm^{-1} Region. Each of the murine interleukin proteins exhibited two IR bands in this region, one at 1686 cm^{-1} and the other at 1674 cm^{-1}. The total percent of band area obtained by summing the two peaks was nearly identical for the α and β proteins (i.e., 21.8 and 19.7, respectively), but the relative fractions were different. Normally, peaks found in this frequency range are assigned to turns, random coils, or β-structures which are not hydrogen-bonded to other peptide units (de Loze et al., 1978). Nevertheless, small peaks can also arise, particularly in the lower part of this region, from various structures (e.g., turns, α-helix) containing CO groups hydrogen-bonded to various amide moieties (Byler & Susi, 1986). A priori, any of these assignments are reasonable; however, for reasons discussed later, these bands were attributed to turns. It is important to realize that IR is very sensitive to secondary structural differences and that these differences reflect the types of turns producing bands for these two proteins. Consequently, while the total number of turns may be the same for rmIL-1α and -1β, they differed in their hydrogen-bonding characteristics.

FT-IR: 1640–1660-cm^{-1} Region. Murine IL-1α and -1β produced two bands between 1640 and 1660 cm^{-1} which accounted for roughly 30% of the total integrated area. For the β form, the peaks were located at 1656 and 1644 cm^{-1}, while the corresponding frequencies observed for the α-form were at 1658 and 1649 cm^{-1}. For both protein species, the band around 1656 cm^{-1} was estimated to represent 20% of the overall protein structure. IR bands occurring between 1640 and 1660 cm^{-1} are usually ascribed to hydrogen-bonded peptide units arising from either α-helices, turns, or random coils (Byler & Susi, 1986) but are also consistent with Na^+ or K^+ salt bridges or CO groups bound to D_2O (Baron et al., 1973; de Loze, 1978). The bands at 1644 and 1649 cm^{-1} in the IL-1 proteins were tentatively ascribed to random coils or CO groups bound to water. For bands close to 1655 cm^{-1}, a good correlation has been established with the presence of α-helices for a large number of proteins (Holloway & Mantsch, 1989; Villalain et al., 1989). However, the CD data clearly provided

no evidence for the presence of α-helices in the rmIL-1 proteins. Recently, Prestrelski et al. (1991a,b) described two protein examples in which a similar IR band around 1655 cm^{-1} was better attributed to large loop structures; one of these proteins, human basic fibroblast growth factor (hbFGF), has recently been found to have similar folding patterns to the interleukins (Zhu et al., 1991). On the basis of the CD data and the precedent mentioned above, the band around 1655 cm^{-1} for the IL-1's was tentatively assigned to extended loops rather than to α-helix structures.

Comparison of IR and CD. As mentioned previously, the CD data provided no evidence of α-helical structure for either form of murine IL-1. The results were generally consistent with those obtained by IR, which indicated that these proteins were primarily composed of β-structures. The percentage of β-structure estimated by CD was 55% for rmIL-1β and 60% for rmIL-1α. When the areas from the three IR bands below 1650 cm^{-1} were added together for IL-1β and -1α, the respective sums were 59.2% and 57.2%. This close agreement for the assignment of β-structure may be factual or it may reflect error in either the CD or IR analysis. In essence, the CD data may be overestimating the amount of β-structure, or a high-frequency IR component of β-strands could have been neglected. If one accepts that the three IR bands correspond to the percent of β-structure estimated by CD, then these results support the assignment of the IR bands near 1647 cm^{-1} to peptide fragments of the β-strands bound to water, rather than to NH groups. Further, the CD data were consistent with the interpretation that ~20% of the overall protein structure contained random or irregular structures. This value corresponded to the percentage of the IR bands near 1655 cm^{-1} that was assigned to large loops rather than to α-helices. Thus, it appeared that the random component obtained by CD was more specifically defined as large loop structures by IR spectra. The number of reverse or tight turns calculated by CD was in good concordance with the IR results.

Comparison with the Human Forms of IL-1. The secondary structural characteristics for the human recombinant forms of IL-1α and -1β (rhIL-1α and -1β) have been studied by NMR and X-ray diffraction. Given the high degree of homology between the respective α and β species, it was useful to compare our IR results with those obtained for the human forms by these other techniques. The secondary structure for rhIL-1β has been investigated by X-ray diffraction (Priestle et al., 1989; Finzel et al., 1989) and by NMR (Driscoll et al., 1990), the latter being in aqueous solutions. In these studies, rhIL-1β contains 12 extended β-strands, 6 of which are arranged in a skewed antiparallel sheet comprising an overall barrel structure. In addition, 8 tight turns or short loops and 3 large loops are present to essentially connect the various β-strands. There is no evidence of α-helices. If one accounts for the total number of amino acids in this protein, the percentages of individual secondary structural components are approximately 27% double β-strands, 29% β-sheets, 20% tight turns, and 24% large loops. These values are in agreement with the IR results as assigned in Table II. All methods indicate about 20% turns, and the NMR data (Driscoll et al., 1990) support the assignment of the 1656-cm^{-1} IR band in the murine IL-1 to large loops. The only apparent discrepancy we found was in the IR assignment of the band at 1644 cm^{-1}, which accounts for 13% of the overall structure. However, if the percentages from this band and the two peaks attributed to the β-strands were summed, a total of 59% β-structure can be achieved for rmIL-1β; this value was essentially similar to the value for the β-structure obtained by NMR (i.e., 56%) for the human form. Therefore, one could argue that the IR band at 1644 cm^{-1} was due to CO groups bound to H_2O or D_2O, depending on the ability of the latter to penetrate the interior of the protein structure. Indeed, other NMR studies (Clore et al., 1990) have reported 11 water molecules which are bound internally for hIL-1β, 8 of which are associated with amino acids in the β-strands, which supported our assignment of this IR band to CO groups bound to water.

The crystal structure of rhIL-1α has also been elucidated recently (Graves et al., 1990) and found to be similar to rhIL-1β. This protein, like the β-form, possesses an overall β-barrel structure with 3-fold symmetry where 12 β-strands constitute the sides and cap of the barrel. In addition to the tight turns and extended loops that are seen, a small 3_{10} helix, similar to the one found in rhIL-1β, is also reported. There is no indication that any part of this IL-1 protein assumes an α-helical conformation. The relative percentages for each type of structure are very similar to that calculated above for rhIL-1β. The IR results, likewise, are in excellent agreement with these X-ray data, further supporting, in this case, the assignment of the 1656-cm^{-1} band to large loops and not to the α-helix. The 3_{10} helix would be expected to absorb near 1639 cm^{-1} (Holloway & Mantsch, 1989) but was not differentiated by the current IR study in either protein. Totaling the IR bands at 1649, 1637, and 1625 cm^{-1} yields a value of 57%, which is very close to the fraction of β-structure (i.e., 54%) predicted by X-ray data. Like the IR data for the β-form, it appears that the peak at 1649 cm^{-1} was due to peptide carbonyls which are bound to water. Lastly, the predominance of the 1637-cm^{-1} band over the 1625-cm^{-1} band, unlike for rmIL-1β, suggests that the β-strands were not associated into a large more stable sheet structure but probably were arranged as double strands.

Concluding Remarks. The IR and CD spectra of rmIL-1α and -1β were determined in aqueous solutions and used to estimate the secondary structures of these proteins. Subsequently, these tentative structural assignments were compared with the definitive architecture of the human forms which had been established by X-ray diffraction or NMR. Together, these findings suggest that the proteins are primarily composed of antiparallel β-structures; albeit there appear to be differences in the degree to which the individual strands are self-associated. In this regard, IR was able to differentiate subtle changes in the types of turns and β-structures. The combined use of IR with CD to determine the secondary structure of most peptides and proteins has shown that an IR band around 1656 cm^{-1} is usually indicative of α-helices. However, in this particular instance, this band must be assigned to a different type of structure. Accordingly, this band was tentatively attributed to large loops connecting some of the β-strands, which was indicated in the CD analysis as random structures. All of the putative assignments made by IR and CD were in good agreement with those results obtained by X-ray and NMR studies. Overall, the use of IR in conjunction with CD appears to be an excellent method to obtain rapid estimates of secondary protein structure for proteins in aqueous solutions. Nonetheless, as seen by these results, IR assignments should be made with extreme caution unless other supportive evidence can be obtained. The real power of infrared spectroscopy lies in the ability to corroborate secondary structure or to follow relative changes in protein structure as a function of selected variables.

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