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Raman spectroscopic analysis of the secondary structure in *Panulirus interruptus* hemocyanin

Robert W. Williams[+] and Wayne A. Hendrickson*

Optical Probes Branch, code 6510, and **Laboratory for the Structure of Matter, code 6030, Naval Research Laboratory, Washington, DC 20375, USA*

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The secondary structure content of *Panulirus interruptus* hemocyanin crystals is estimated to be 29 ± 5% helix and 42 ± 4% β-strand. These estimates are obtained from an analysis of the Raman amide I spectrum. This report constitutes a test of this method of analyzing Raman spectra since the X-ray structure of *P. interruptus,* which is unknown to us, is under intensive study. Also, the amide I spectrum of *P. interruptus* in solution, at a somewhat higher pH, is significantly different from the spectrum of crystals which indicates that the secondary structure content may be different in the solution sample.

Secondary structure *Raman spectroscopy* *Hemocyanin*

1. INTRODUCTION

A new method for estimating protein secondary structure from the amide I band in Raman spectra has recently been described [1]. The reliability of this method has been estimated by comparing its results with those obtained from X-ray diffraction for a number of reference proteins whose structures are known. Bias towards the known structures has been avoided by excluding each protein from the reference set when its structure is calculated. However, it is desirable to corroborate these results by accurately predicting the conformational composition of a protein before its X-ray structure is known. Toward this goal, we have undertaken to determine the secondary structure of *Panulirus interruptus* hemocyanin prior to our knowledge of an imminent interpretation of the crystal structure at 3.2 Å resolution (Hol, W.G.J. private communication).

Hemocyanins are oxygen-transport proteins found freely dissolved in the hemolymph of certains arthropods and molluscs. Properties of the oxygen-binding copper centers are similar in all hemocyanins, but the proteins, while always large and complex, differ markedly in molecular architecture between the two phyla [2,3]. The simplest of the arthropodan hemocyanin is that of the spiny lobster, *P. interruptus.* It is a 450-kDa hexamer [4] that has approximate D_3 symmetry [5]. The molecules appear to be composed from a non-stoichiometric mixture of at least 5 components [6]. The subunit composition in the crystals under study [5] is similar to that in the hemolymph [6]. The crystal structure has been described at 5 Å resolution [5] and a preliminary account of results at 4 Å resolution has also been given [7] where each subunit is described as containing a β-barrel as well as a large number of α-helices.

Circular dichroism (CD) spectra of other arthropod hemocyanins have yielded conflicting estimates of helix content. Results from one study [8] indicated about 14% helix for *Carcinus maenas*

[+] Present address: Biochemistry Department, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, USA

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and *Limulus polyphemus,* while CD spectra of *Jasus edwardsii* hemocyanin [9] are typical of proteins containing 30–40% helix, by our estimate.

2. MATERIALS AND METHODS

Crystals of *P. interruptus* hemocyanin were essentially the same as those described for the X-ray work in [5]. The mother liquor was 0.01 M HOAc/NaOAc buffer, pH 4.5. Crystals were supplied by W.G.J. Hol on two different occasions, August 1982 and June 1983, on which separate experiments were run.

In each experiment 4–6 crystals were placed at the bottom of a 0.7 mm diameter quartz capillary filled with mother liquor. The capillary was placed in a thermostated sample holder set to 10°C. The crystals were irradiated with about 100 mW of laser light focussed to a beam about 0.5 mm in diameter at the sample.

Lyophilized *P. interruptus* hemocyanin prepared by Johan Vereyken from a 2% protein, 5% sucrose, 50 mM Tris, 5 mM EDTA (pH 8.5) solution was also supplied by W.G.J. Hol. This material was exhaustively dialyzed in 50 mM Tris, 10 mM $CaCl_2$ (pH 7.2) to remove the sucrose. The hemocyanin is hexameric under these conditions. The final protein concentration was about 5%. About 10 μl of this solution were placed in a melting point capillary, thermostated to 10°C. and irradiated with about 300 mW of laser light during collection of the Raman spectrum.

The Raman instrumentation, reference proteins, and method of data analysis for structure information are the same as in [1] with 3 minor exceptions. First, the spectral region used here for structure calculations is from 1630 to 1710 cm^{-1}, instead of only to 1700 cm^{-1}. Second, the reference set includes avidin secondary structure estimates as reported in [10]. Third, an alternative criterion for the subtraction of the water band at 1640 cm^{-1} was tested and found to give the same results as the method in [11]. This criterion was used for the spectra of crystalline samples and is described below.

Proteins do not give Raman bands between 1720 and 1800 cm^{-1}. Spectra of dry proteins with little fluorescence show a linear baseline in this region. However, the water band at 1640 cm^{-1} has considerable intensity at 1720 cm^{-1} and shows curvature from 1720 to 1800 cm^{-1}. The spectrum of water, which extends under the amide I band, is subtracted from an aqueous protein spectrum so that the region between 1720 and 1800 cm^{-1} is nearly linear.

3. RESULTS

Raman spectra of crystals and a solution of *P. interruptus* hemocyanin are shown in fig. 1. The amide I bands from these samples are significantly different between 1630 and 1685 cm^{-1}. An attempt to account for this difference in terms of water subtraction or baseline adjustment artifacts was made. Changes in these variables never reduced the difference observed in fig. 1.

The effect of errors in water subtraction on the secondary structure estimates was tested. The maximum error introduced through water subtraction of noisy spectra appears to be ±1 SD in the structure estimates.

Estimates of secondary structure content calculated from these spectra are listed in table 1. Values for the total helix and β-strand content in the crystalline state are about 2 SD different from the values obtained for the solution. Thus, an

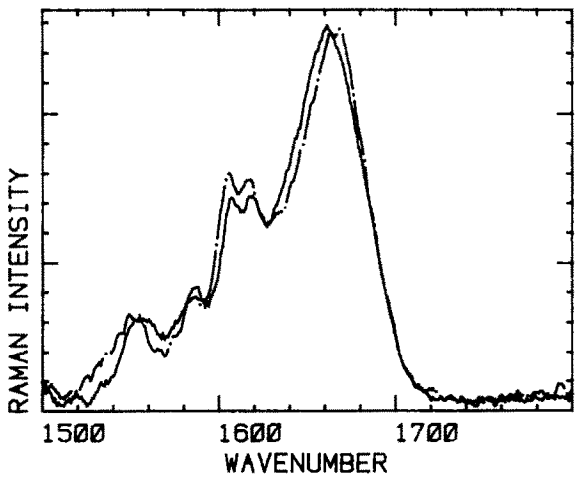

Fig. 1. Raman amide I spectra of *P. interruptus* hemocyanin in crystals (-----) and solution (——). Bands below 1630 cm^{-1} are due to aromatic side chains. The solution spectrum is unsmoothed data. The crystal spectrum has been 7 point smoothed as in [13].

Table 1

Percent of secondary structure content in *P. interruptus* hemocyanin as estimated from Raman amide I spectra

State	Structure type[a]						Totals		Method[b]
	Ho	Hd	Sa	Sp	T	U	H	S	
Crystals pH 4.5	18	11	40	1	19	11	29	42	R1
	20	8	40	1	18	13	28	41	R2
Solution pH 7.2	26	14	31	0	17	12	39	32	R1
	26	10	33	0	17	14	36	33	R2
Standard deviation	4	4	3	4	2	2	5	4	R1
	5	4	4	4	2	3	4	3	R2

[a] Ho, ordered α-helix; Hd, disordered helix; Sa, antiparallel β-strand; Sp, parallel β-strand; T, turn; U, undefined; H, total helix; S, total β-strand

[b] R1, using a non-negative least squares procedure; R2, using a singular value analysis as in [1]

hypothesis that the secondary structure content of crystalline and solution states is the same could be rejected at about the 0.05 level of significance.

4. DISCUSSION

The difference between spectra of crystalline and solution states is surprising and perhaps unprecedented. A small difference has been previously seen between insulin crystals at pH 7 and insulin in solution at pH 2 [11], but secondary structure content was calculated to be the same in this case. Other examples exist in [10,12] where the amide I spectra of proteins in crystals and in solution are exactly identical.

This evidence suggests that there is a difference in the secondary structure compositions of the samples we have studied. The difference in pH, or the way crystals form from the mixed solution of different subunits may account for changes in the conformational content of the samples.

The β-sheet in *P. interruptus* is predicted here to be entirely antiparallel. This is significant insofar as the method we have used gives predictions of parallel β-sheet which correlate positively with actual values [1]. However, the number of proteins with significant parallel β-sheet content correctly predicted is not large, so there is only weak evidence here that the β-sheet in *P. interruptus* is antiparallel. Moreover, the worst prediction that has been made using the Raman amide I method is for a parallel α/β protein, triosephosphate isomerase, where estimates are wrong by about 30% in the discrimination between parallel and antiparallel β-sheet [1]. Therefore, we regard the parallel β-sheet analysis from this method as being of interest, but not completely developed. Otherwise, the worst predictions that have been obtained [1] for total β-sheet or helix using the R1 method (table 1) are in error by only 10%, or 2 SD.

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REFERENCES

[1] Williams, R.W. (1983) J. Mol. Biol. 166, 581–603.
[2] Hendrickson, W.A. (1977) Trends Biochem. Sci 2, 108–111.
[3] Van Bruggen, E.F.J. (1980) Trends Biochem. Sci. 5, 185–188.
[4] Kuiper, H.A., Gaastra, W., Beintema, J.J., Van Bruggen, E.F.J., Schepman, A.M.H. and Drenth, J. (1975) J. Mol. Biol. 99, 619–629.
[5] Van Schaik, E.J.M., Schutter, W.G., Gaykema, W.P.J., Schepman, A.M.H. and Hol, G.W.J. (1982) J. Mol. Biol. 158, 457–485.

[6] Van Eerd, J.P. and Folkerts, A. (1981) in: Invertebrate Oxygen-Binding Proteins (Lamy, J. and Lamy, J. eds) pp. 139–149, Marcel Dekker, New York.
[7] Gaykema, W.P.J., Van Schaick, E.J.M., Schutter, W.G. and Hol, W.G.J. (1983) in: Life Chemistry Reports Suppl. 1 (Wood, E.J. ed.) pp. 57–60.
[8] Tamburro, A.M., Salvato, B. and Zatta, P. (1976) Comp. Biochem. Physiol. 55B, 347–356.
[9] Ellerton, H.D., Collins, L.B., Gale, J.S. and Yung, A.Y.P. (1977) Biophys. Chem. 6, 47–57.
[10] Honzatko, R.B. and Williams, R.W. (1982) Biochemistry 24, 6201–6205.
[11] Williams, R.W. and Dunker, A.K. (1981) J. Mol. Biol. 152, 783–813.
[12] Williams, R.W., Gaber, B.P. and Gunning, J. (1982) J. Biol. Chem. 257, 13321–13323.
[13] Savitsky, A. and Golay, M. (1967) Anal. Chem. 36, 1627–1639.