Bevington, P. R. (1969) in *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.
Bjorkhem, I., Danielsson, H., & Wikvall, K. (1974) *J. Biol. Chem. 249*, 6439–6445.
Brewer, J. M. (1974) in *Experimental Techniques in Biochemistry* (Brewer, J. M., Pesce, A. J., & Asworth, R. B., Eds.) p 3, Prentice-Hall, Englewood Cliffs, NJ.
Edwards, K., Fleischer, B., Dryburgh, H., Fleischer, S., & Schreiber, G. (1976) *Biochem. Biophys. Res. Commun. 72*, 310–318.
Fleischer, B. (1974) *Methods Enzymol. 31*, 180–191.
Fleischer, B. (1981) *Arch. Biochem. Biophys. 212*, 602–610.
Fleischer, S., & Fleischer, B. (1967) *Methods Enzymol. 10*, 406–433.
Fleischer, S., & Kervina, M. (1974) *Methods Enzymol. 31*, 6–41.
Goldsmith, M. A., Murling, S., & Jones, A. L. (1983) *Gastroenterology 84*, 978–986.
Helenius, A., McCaslin, D. R., Fries, E., & Tanford, C. (1979) *Methods Enzymol. 56*, 734–749.
Jones, A. L., Schmucker, D. L., Mooney, J. S., Ockner, R. K., & Adler, R. D. (1979) *Lab. Invest. 40*, 512–517.
Killenberg, P. G., & Jordan, J. T. (1978) *J. Biol. Chem. 253*, 1005–1010.
Kramer, W., Bickel, U., Buscher, H. P., Gerok, W., & Kurz, G. (1982) *Eur. J. Biochem. 129*, 13–24.
Kuron, G. W., & Tennent, D. M. (1961) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 20*, 268a.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mingrone, G., & Greco, A. V. (1980) *J. Chromatogr. 183*, 277–286.
Okishio, T., & Nair, P. P. (1966) *Biochemistry 5*, 3662–3668.
Peters, T., Jr., Fleischer, B., & Fleischer, S. (1971) *J. Biol. Chem. 246*, 240–244.
Roda, A., Cappetteri, G., Aldini, R., Roda, E., & Barbara, L. (1982) *J. Lipid Res. 23*, 490–495.
Sandberg, D. H., Sjovall, J., Sjovall, K., & Turner, D. A. (1965) *J. Lipid Res. 6*, 182–192.
Schwarz, L. R., Burr, R., Schwenk, M., Pfaff, E., & Greim, H. (1975) *Eur. J. Biochem. 55*, 617–623.
Shaw, R., Smith, J. A., & Elliott, W. H. (1978) *Anal. Biochem. 86*, 450–456.
Simion, F. A., Fleischer, B., & Fleischer, S. (1983a) *Biochemistry 22*, 5029–5034.
Simion, F. A., Fleischer, B., & Fleischer, S. (1983b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2021a.
Simion, F. A., Fleischer, B., & Fleischer, S. (1983c) *J. Cell Biol. 97*, 305a (Abstr.).
Strange, R. C., Nimmo, I. A., & Percy-Robb, I. W. (1976) *Biochem. J. 156*, 427–433.
Strange, R. C., Nimmo, I. A., & Percy-Robb, I. W. (1977) *Biochem. J. 162*, 659–664.
Strange, R. C., Beckett, G. J., & Percy-Robb, I. W. (1979a) *Biochem. J. 178*, 71–78.
Strange, R. C., Chapman, B. T., Johnston, J. D., Nimmo, I. A., & Percy-Robb, I. W. (1979b) *Biochim. Biophys. Acta 573*, 535–545.
Sugiyama, Y., Yamada, T., & Kaplowitz, N. (1983) *J. Biol. Chem. 258*, 3602–3607.
Tanford, C. (1980) in *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, p 91, Wiley, New York.
Turley, S. D., & Dietschy, J. M. (1978) *J. Lipid Res. 19*, 924–928.
Vessey, D. A. (1979) *J. Biol. Chem. 254*, 2059–2063.
Wilson, F. A., & Treanor, L. L. (1977) *J. Membr. Biol. 33*, 213–230.
Zambrano, F., Fleischer, S., & Fleischer, B. (1975) *Biochim. Biophys. Acta 380*, 357–369.

Exciton Interaction in Allophycocyanin†

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ABSTRACT: The absorption and circular dichroism (CD) spectra of allophycocyanin II in the trimer and monomer (dissociated) forms were resolved into four and two components, respectively. The short-wavelength region of the visible spectra was approximated by a chimera of Lorentzian- and Gaussian-shaped bands having a bandwidth of ca. 65 nm. The rest of the bands have a pure Gaussian form. The characteristic 652-nm band in the absorption spectrum (656 nm in the CD spectrum) is shown to arise from exciton interaction between two fluorescent phycocyanobilin chromophores.

Allophycocyanin (APC) is that biliprotein that occupies the core of the light-harvesting phycobilisome and is in close association with the thylakoid membrane. It has been shown to exist in four spectral forms, APC I, II, III, and B (Gantt, 1981). From spectroscopic studies it has been inferred that APC II and III transmit excitation energy to the terminal emitters APC I and B (Gantt, 1980). The spectral characteristics of APC were discussed by MacColl et al. (1980), and the conclusion was drawn that the 652-nm absorption band of the trimer was due to intermediate-strength interaction between its chromophores. Allophycocyanin is composed of two subunits, α and β, each containing a single phycocyanobilin chromophore (Brown et al., 1975).

The absorption spectra of the individual APC trimers have been resolved into several Gaussian curves by computer analysis (Zilinskas et al., 1980). The absorption spectra of allophycocyanin "trimer majority" and "monomer majority" have also been similarly analyzed (Mimuro et al., 1982). In

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the spectral region common to both deconvolutions (500–700 nm), eight and seven Gaussian bands were obtained for APC II and III, respectively, by Zilinskas et al. (1980) and nine bands for both the APC "trimer" and "monomer" by Mimuro et al. (1982).

The present paper reports on the computer-assisted deconvolution of APC II absorption and circular dichroism spectra, both in the purely trimer and monomer forms. Care was taken to base analysis on the spectral features and the known chromophore composition of six phycocyanobilins for the trimer and two for the monomer. This represents the first deconvolution of the CD spectrum of any biliprotein.

Materials and Methods

Allophycocyanin isolation and purification were carried out using a considerably modified version of the procedures described by Zilinskas et al. (1978) and MacColl et al. (1981). Ten grams of *Anacystis nidulans* cells was suspended in 30 mL of pH 7.5, 50 mM Tris-HCl buffer and disrupted by sonication (40 cycles of 15 s in an ice bath). An equal volume of buffer was then added to the cell suspension, which was further treated with 1% Triton X-100 (Sigma) for 30 min. The sample was centrifuged at 15000 rpm in a Sorvall SS-34 rotor (29000*g*) for 20 min and the supernatant treated with 35% $(NH_4)_2SO_4$ for 1 h and spun down at 15000 rpm for 20 min. The sediment was resuspended in 100 mL of pH 7.0, 1 mM H_3PO_4/KOH buffer containing 100 mM NaCl and loaded onto a 7.5 × 4.0 cm hydroxylapatite (Bio-Rad) column equilibrated with 10 volumes of the buffer. Phycocyanin was eluted from the column by using 100 mM, pH 7.0, H_3PO_4/KOH buffer containing 100 mM NaCl. The remaining allophycocyanin was eluted by using 200 mM, pH 7.0, H_3PO_4/KOH buffer containing 100 mM NaCl. Five-milliliter fractions were collected. Allophycocyanin II was collected as fractions with an A_{620}/A_{650} ratio of 0.59–0.63 (Zilinskas et al., 1978). All operations were carried out at room temperature. Allophycocyanin monomers were prepared according to MacColl et al. (1981) using $NaClO_4$. For spectroscopic measurements APC was dialyzed into pH 7.0, 0.1 M sodium phosphate buffer.

CD measurements were carried out using a Cary 61 spectropolarimeter. Typical samples of allophycocyanin measured had an optical density of 1 in a 2 mm path length cuvette. CD spectra were read at 1-nm intervals, and data were manually entered into an Apple computer for storage.

The absorption spectra were obtained with a Perkin-Elmer 320 absorption spectrophotometer. The spectra were sampled at 1-nm intervals and the data collected via a RS232-C interface between the photometer and an Apple II computer and stored on a floppy diskette for later analysis.

A curve-fitting program called FITGAUSS was developed to approximate the experimental curve with the sum of a given number of Gaussian curves by varying the amplitude, the half-bandwidth, and the wavelength of the band maximum. Since the experimental data were obtained on a wavelength scale whereas the component curves are calculated on the basis of a wavenumber scale, care was taken to calculate the component curves at values where experimental data were available. The resulting Gaussian curves appear skewed toward the red when plotted on the wavelength scale, but this technique avoided the introduction of additional errors into the fitting procedure inherent in any smoothing or transformation of experimental data.

The half-bandwidths of the Gaussian curves were taken at half of the maximal amplitude. Chimeric components were obtained by grafting a Lorentzian curve to a Gaussian curve at their maxima, the two curves having equal amplitudes at their peak and the same width at half-amplitude.

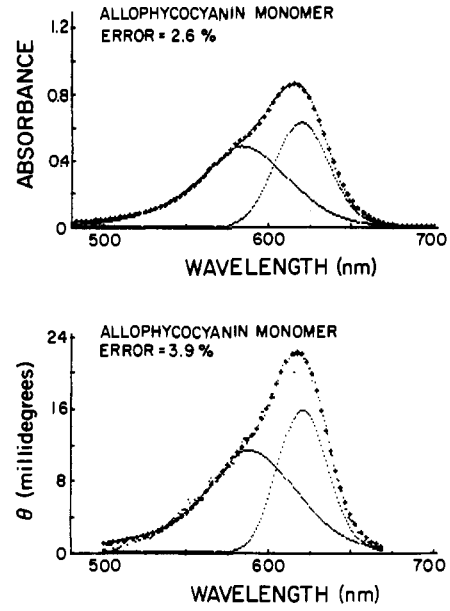

FIGURE 1: Computer-assisted deconvolution of the absorption and CD spectra of allophycocanin monomers in the presence of 1 M $NaClO_4$ at pH 7.0. The optical path length is 1 cm. The experimental points and the component curves are indicated with dots (···) and the calculated sum of the components is shown as crosses (+). For the sake of clarity, only every third point in the sum curve is presented.

In all cases the chimeric components consisted of a Lorentzian-shaped high-energy half and a Gaussian-shaped low-energy half.

The fitting procedure minimizes the root mean squares of the displacement of the fitted curve from the experimental curve and characterizes the error of the fit by calculating the ratio of the sum of the root mean square displacements to the area under the experimental curve. This value is given as the error and is always greater than the difference between the areas of the experimental and fitted curves, which came to less than 1%.

Results and Discussion

In analyzing the absorption spectra of biliproteins, it was found that, while the long-wavelength edge can be well approximated by a Gaussian curve, the short-wavelength edge is best described by a Lorentzian curve. This is demonstrated in the present paper for the case of allophycocyanin. Figures 1 and 2 show the results of deconvolution for both the monomer and trimer spectra into two and four components, respectively. Absorption and CD spectra are shown together to demonstrate the one to one correspondence of resolved bands between them. Quantitative data are given in Table I.

An important feature of these spectra is the shortest wavelength component. It consists of a Lorentzian curve spanning the short-wavelength region up to the peak (λ_{max} = 603 and 585 nm for the trimer and monomer, respectively) and continues in a Gaussian-shaped drop toward the red part of the spectrum. The bandwidth at half-height of both curves is the same. It is evident from the figures that the Gaussian-shaped bands present at higher wavelengths have only a negligible contribution in the spectral region where the Lorentzian curve dominates.

The rationale for this chimeric component is found by invoking the Franck–Condon principle. According to this principle, an electronic transition occurs between the ground and excited states of a molecule while the nuclear coordinates

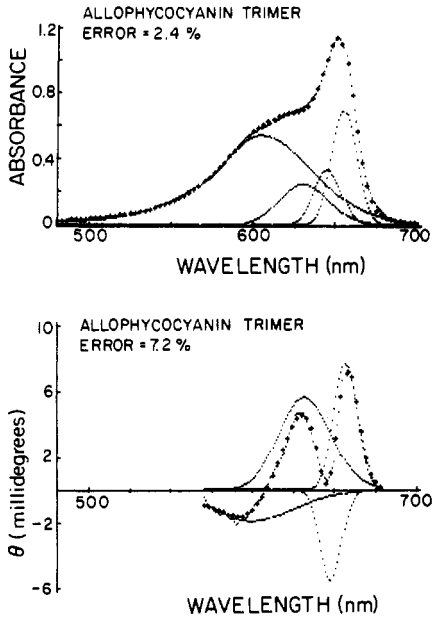

FIGURE 2: Computer-assisted deconvolution of the absorption and circular dichroism spectra of allophycocyanin trimers at pH 7.0. The optical path length is 0.2 cm, the protein concentration being 5 times that in Figure 1 giving the same quantity of protein measured. The experimental points and the component curves are indicated with dots (···) and the calculated sum of the components is shown as crosses (+). For the sake of clarity, only every third point in the sum curve is presented.

Table I: Data Obtained by the Computer-Assisted Deconvolution of Allophycocyanin Absorption and Circular Dichroism Spectra into Chimeric (Lorentzian/Gaussian) and Gaussian Components[a]

APC form		wavelength maximum (nm)	half-width (nm)	oscillator strength	relative area
monomer	absorption	585.5	65.2	2.49	
		619.4	36.9	1.56	
	CD	585.6	59.3		40
		619.5	35.2		33.5
trimer	absorption	603.7	68	2.91	
		632.2	34.2	0.546	
		646.6	18.9	0.385	
		655	18.6	0.7	
	CD	598.1	61.3		5.5
		631.3	37.3		11
		647.8	16.7		4.5
		656.8	15.7		6.7

[a] Oscillator strengths were calculated by assuming a molecular extinction coefficient of 90 000 per phycocyanobilin chromophore based on Bryant et al. (1976). Relative areas are given for the CD bands due to the ambiguity of molecular ellipticity for allophycocyanin (see the text).

are assumed to remain unchanged. The transition, however, may involve a varying number of vibronic levels depending on the mutual displacement relative to each other of the two states (Turro, 1965). A transition to a single vibronic level will cause the absorption band to assume a Gaussian shape due to thermal vibrations and Doppler broadening (Kauzman, 1957). If the transition involves several vibronic bands, a wide absorption band is expected and Figures 1 and 2 imply that a progression of vibronic bands belonging to a single electronic transition may be approximated by a Lorentzian-shaped envelope. However, if the electronic transition is centered around a vibronic level with the lowest possible quantum number, it is reasonable to assume that the line shape on the low-energy side of the maximum will be governed by a single vibronic level and therefore will be approximated by a Gaussian curve.

Thus, an absorption band centered in the vicinity of a 0–0 transition and enveloping a series of vibronic transitions is expected to assume a chimeric shape discribed above. In fact, were the shortest wavelength components in Figures 1 and 2 pure Lorentzian curves they would not fit under the experimental curves in the long-wavelength region.

It is understood that the sum of a series of Gaussian-shaped curves can mathematically just as well approximate the high-energy region of allophycocyanin absorption, and in fact a 560- and 502-nm "supplemental band" (Mimuro et al., 1982) was introduced "for better fitting". We maintain that at present there are insufficient data at hand to allow an unequivocal assignment of separate Gaussian curves to individual vibronic levels in allophycocyanin. This conclusion is supported by the fact that three different deconvolutions were obtained for the APC trimer absorption spectrum using four components having approximately the same half-bandwidths as those in Table I and one deconvolution using three components. All three four-component deconvolutions accounted for an exciton split in the allophycocyanin trimer, albeit at different wavelength maxima for the high-energy component, but only one (Figure 2) proved to be compatible with the deconvolution of the CD spectrum of the allophycocyanin trimer. A unique solution is thus provided by the simultaneous deconvolution of both the absorption and CD spectra.

The absorption spectrum of the allophycocyanin monomer (Figure 1) can be resolved into two components with peak wavelengths at 585 and 619 nm. Since each allophycocyanin monomer consists of an α and a β subunit having one covalently attached phycocyanobilin chromophore (Brown et al., 1975), the two bands are assumed to represent these chromophores. In going from the monomer to the trimer form, the spectra undergo considerable changes, the salient feature being the emergence of a sharp peak at 652 nm in the trimer (Figure 2). The change is reversible (MacColl et al., 1980) and allows the two figures to be discussed in such a context. The chimeric component shifts from 585 to 603 nm. The three remaining bands in the trimer are assumed to arise from the 619-nm component in the monomer spectrum and represent three phycocyanobilin chromophores. Two of these chromophores form a dimer and enter into exciton interaction that leads to a split in energy levels (647 and 655 nm) and is well documented by the CD spectrum that exhibits a corresponding pair of a negative component at 648 nm and a positive one at 657 nm. The third chromophore at 632 nm underwent only a red shift due to chromophore–protein interaction and manifests itself as a positive component at 631 nm in the CD spectrum.

These results suggest that the assembly of monomers to trimers (MacColl et al., 1980, 1981) consists of two chromophore-related events, an exciton split and a change in protein–chromophore interactions. One possible result of a change in protein–chromophore interaction is an alteration in the chromophore conformation and subsequent change in the spectral properties (Scheer et al., 1982).

Table I shows the correspondence in band maxima and half-widths. The oscillator strength of the chimeric component (585 nm) changes about 20%, from a value of 2.5 for three chromophores (0.83 each) to 2.9. The changes in oscillator strengths on the other subunit are more interesting, however, from 0.52 to 0.54 for the noninteracting chromophore and to 0.385 and 0.7 for the remaining two interacting chromophores. The total oscillator strength of the three long-wavelength chromophores is conserved but unevenly distributed. The values for the interacting chromophore pair allow the calculation of the angle between the transition moments in the

chromophore dimer using the expression

$$D_{A\pm} = D_a \bullet D_a \cos\theta$$

(Cantor & Schimmel, 1980). This gives an angle of approximately 72°, where D_A is the chromophore pair dipole strength and D_a is the dipole strength of the individual transitions constituting the exciton pair.

In contrast to the relatively minor change in total absorption intensity between the monomer and trimer forms, the area under the CD spectrum of the trimer is considerably less than in the case of the monomer. Canaani & Gantt (1980) have published the CD spectrum of the APC II trimer and have pointed to the possibility that the 632- and 656-nm peaks correspond to environmentally different chromophores. Whereas this is essentially correct, in the present paper it is shown that two more, negative, bands also contribute to the CD spectrum of the trimer. This provides an explanation for the fact that the observed CD bands have significantly diminished half-widths compared to what might be expected on the basis of the absorption spectrum.

The contribution from the chimeric band is 40 relative units in the monomer, and it drops to 5.5 relative units in the trimer. Thus, the unequivocal assignment of a value for the molecular ellipticity in order to calculate the rotational strength of the individual components was not attempted here, and Table I contains the relative areas of the bands in arbitrary units. The single noninteracting chromophore at 631 nm retains its contribution ($^1/_3$ of that in the monomer for three chromophores) just as in the absorption spectrum, lending further support for the model implied by the above discussion. The negative CD band at 647.8 nm is suggested by the steep drop (Figure 2) in the measured spectrum from 656 nm toward the blue, and its presence in the deconvolution is evidence for the spectral split due to the exciton pair.

Dale & Teale (1970) and Teale & Dale (1970) introduced the terminology of fluorescing and sensitizing chromophores, f and s, respectively, explaining the fluorescence polarization spectra of the phycobiliproteins phycocyanin and phycoerythrin. Deconvolution of the monomer spectra of allophycocyanin into two components is in harmony with fluorescence polarization data that show a a stepwise increase in the degree of polarization (MacColl et al., 1978, 1980) in the region where the 619-nm component begins to dominate the absorption spectrum. This result allows the assignment of the 585-nm band to sensitizing and the 619-nm band to fluorescing chromophores. The fluorescence polarization spectrum of the allophycocyanin trimer is a featureless straight line and indicates that almost complete depolarization occurs (3–5%). This is due to the electronic excitation energy being transferred from the s chromophores to the f chromophores and subsequent excitation delocalization on the exciton pair as well as the third f chromophore that probably is situated close to the exciton pair.

The theoretical treatment of the exciton model by Kasha et al. (1965) based on the Simpson & Peterson (1957) criteria for the strength of the intermolecular vibrational–electronic interaction between excited states relative to the strength of intramolecular vibrational–electronic coupling allows the evaluation of spectral data for allophycocyanin. In the strong coupling case the relation $2U/\Delta E \gg 1$ holds whereas in the weak coupling case the inequality $2U/\Delta E \ll 1$ is proposed, where $2U$ is the exciton interaction energy and ΔE is the Franck–Condon bandwidth of the corresponding molecular electronic transition in the individual molecular unit.

For allophycocyanin the exciton split between the interacting pair is $2U \simeq 210$ cm^{-1} and the ΔE bandwidth is $\simeq 900$ cm^{-1}.

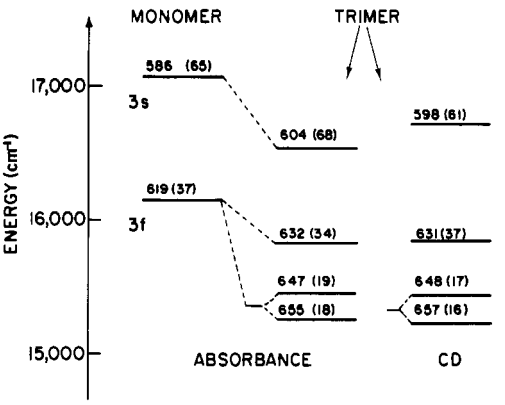

FIGURE 3: Energy level diagram of allophycocyanin based on data obtained from the resolution of absorption and circular dichroism spectra. The three-digit numbers stand for the wavelength maxima; the numbers in parentheses indicate the half-bandwidth of the components. 3s and 3f stand for sensitizing and fluorescent chromophores, respectively, in the monomer.

The relation $2U/\Delta E$ gives 0.25, placing the interaction in or just short of the intermediate-strength range.

In summary, the resolution of the allophycocyanin absorption and CD spectra into the same four components satisfactorily accounts for the known chromophore composition (six phycocyanobilins) of the pigment molecule. A chimeric component at short wavelengths represents three sensitizing chromophores. The characteristic 652-nm band in the absorption spectrum is a composite of two components, one due to the low-energy (656-nm) transition and the other the high-energy (646-nm) transition of an exciton interaction between two chromophores (Figure 3). These, along with the 631-nm chromophore, are designated as fluorescent chomophores in the Teale & Dale (1970) terminology. Although a model based on strictly conformational changes cannot be ruled out, evidence for the exciton pair is further borne out by the bisignate character of the deconvoluted CD spectrum with a positive band at 657 nm and a negative band at 647.8 nm.

References

Brown, A. S., Foster, J. A., Voynow, P. V., Franzblau, C. F., & Troxler, R. F. (1975) *Biochemistry 14*, 3581–3588.

Bryant, D. A., Glazer, A. N., & Eiserling, F. A. (1976) *Arch. Microbiol. 110*, 61–75.

Canaani, O. D., & Gantt, E. (1980) *Biochemistry 19*, 2950–2956.

Cantor, C. R., & Schimmel, P. R. (1980) in *Biophysical Chemistry*, p 395, W. H. Freeman, San Francisco.

Dale, R. E., & Teale F. W. J. (1970) *Photochem. Photobiol. 12*, 99–117.

Gantt, E. (1980) *Int. Rev. Cytol. 66*, 45–80.

Gantt, E. (1981) *Annu. Rev. Plant Physiol. 32*, 327–347.

Glazer, A. N., Fang, S., & Brown, D. M. (1973) *J. Biol. Chem. 248*, 5679–5685.

Kasha, M., Rawls, H. R., & Ashraf el Bayoumi, M. 1965) *Pure Appl. Chem. 11*, 371–392.

Kauzman, W. (1957) in *Quantum Chemistry*, p 566, Academic Press, New York.

MacColl, R., Edwards, M. R., & Haaksma, C. (1978) *Biophys. Chem. 8*, 369–376.

MacColl, R., Csatorday, K., Berns, D. S., & Traeger, E. (1980) *Biochemistry 19*, 2817–2820.

MacColl, R., Csatorday, K., Berns, D. S., & Traeger, E. (1981) *Arch. Biochem. Biophys. 208*, 42–48.

Mimuro, M., Murakami, A., & Fujita, Y. (1982) *Arch. Biochem. Biophys. 215*, 266–273.

Scheer, H., Formanek, H., & Schneider, S. (1982) *Photochem. Photobiol. 36*, 259–272.
Simpson, W. T., & Peterson, D. L. (1957) *J. Chem. Phys. 26*, 588–593.
Teale, F. W. J., & Dale, R. E. (1970) *Biochem. J. 116*, 161–169.
Turro, N. S. (1965) in *Molecular Photochemistry*, pp 35–43, W. A. Benjamin, Reading, MA.
Zilinskas, B. A., Zimmerman, B. K., & Gantt, E. (1978) *Photochem. Photobiol. 27*, 587–595.
Zilinskas, B. A., Greenwald, L. S., Bailey, C. L., & Kahn, P. C. (1980) *Biochim. Biophys. Acta 592*, 267–276.

Nonaromatic Amino Acids in the Combining Site Region of a Monoclonal Anti-Spin-Label Antibody†

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ABSTRACT: The nuclear magnetic resonance spectra of monoclonal Fab antibody fragments have been recorded in the absence and presence of the specific spin-label dinitrophenyl hapten. The difference spectra reveal the presence of about 50 amino acids in the region of the combining site. By selective deuteration and by use of double difference spectra, all the resonances in the spectral region −1 to 1.5 ppm have been identified. We have found that in the combining site region there are four or five valines, certainly three and possibly five threonines, three or four leucines, two or three isoleucines, and six or seven alanines. Selective deuteration of methionine and lysine reveals one methionine and two lysines in the difference spectra. All of these amino acids are estimated to be within 17 Å of the paramagnetic hapten. By using difference spectra involving low fractional occupancy of the combining site with the spin-label hapten, it is established that one threonine and one valine are very close to the paramagnetic hapten.

Much progress has been made in accounting for the diversity and specificity of antibodies through studies of amino acid sequences and immunoglobulin genetics. X-ray crystallographic studies have shown that the hypervariable loops are responsible for binding site structure and that the more highly conserved sequences in the variable region are responsible for forming the immunoglobulin fold, the structural motif upon which all variable regions are built (Wu & Kabat, 1970; Amzel & Poljak, 1979). The number of antibody–hapten complexes studied by X-ray crystallography has been far too small to enable one to predict combining site structure and specificity from the amino acid sequence. It is with this and related problems in mind that we have undertaken a nuclear magnetic resonance study of a monoclonal antibody and its interactions with the parmagnetic hapten that it is directed against.

Previous workers (Dower & Dwek, 1979) have shown that it is possible to use the differences between the NMR spectra of the antibody and the antibody–hapten complex to get information about the structure of the binding site. We have extended this concept by employing the broadening effect of the spin-label hapten and biosynthetic incorporation of deuterated amino acids to identify the aromatic amino acid present in the combining site region of our monoclonal AN02 antibody (Anglister et al., 1984a). We have also shown that hapten exchange is fast enough that titration of the binding site (Campbell et al., 1975) allows the determination of proton–spin-label distances for well-resolved resonances in the difference spectra (Anglister et al., 1984b).

In the present work, we have used these approaches to study the more complicated spectral region due to the nonaromatic amino acids. The complexity of the spectrum in this region makes it necessary to use double difference spectra. In this type of analysis, the normal difference spectra (without hapten minus with hapten) are calculated for Fab's that differ only in the incorporation of one selected deuterated amino acid. The two difference spectra are then subtracted, leaving only the contribution of the selected amino acid in the double difference spectrum. We have assigned a large number of the resonances in the difference spectrum by this technique and in some favorable cases have made estimates of the distances between the amino acids and the paramagnetic hapten. It is hoped that such information, together with the (as yet unknown) amino acid sequence, will lead to a useful model of the combining site. Our present work and previous work on this problem clearly indicate that there are significant structural changes upon hapten binding.

Materials and Methods

The synthesis of the spin-label hapten has been described (Balakrishnan et al., 1982). The chemical formula can also be found in Anglister et al. (1984a).

The origin, maintenance, and labeling of the AN02 cell line have been described previously (Anglister et al., 1984a). Fab fragments were prepared by standard procedures. The amino acids perdeuterated L-lysine, L-threonine, and L-isoleucine were purchased from Cambridge Isotopes Laboratories. Perdeuterated L-alanine, L-leucine, L-valine, and L-[*methyl*-$^2H_3$]methionine were purchased from MSD Isotopes. Medium labeled with deuterated alanine included 3–4 mM alanine. In all other cases, the amino acids were in the regular quantities for RPMI medium. NMR spectra were taken with a JEOL 500-MHz spectrometer. The concentration of Fab was in the range (1.3–3) × 10^{-4} M, and sample volumes were 550 μL. Free induction decays were collected in 8000 data points after

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