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On the Origin of the Positive Band in the Far-ultraviolet Circular Dichroic Spectrum of Fibronectin*

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M. Yahiya Khan‡, German Villanueva§, and Stuart A. Newman

From the Departments of Cell Biology and Anatomy and §Biochemistry, New York Medical College, Valhalla, New York 10595

The genesis of the positive bands in the far-ultraviolet circular dichroic spectra of human plasma fibronectin and its 31-kDa NH_2-terminal heparin-binding fragment was studied. Spectra of ester derivatives of tyrosine, tryptophan, and phenylalanine and of model mixtures of these derivatives in which they are present in the same ratios as in the proteins indicate that all the aromatic side chains make substantial contributions to composite positive bands with maxima several nanometers below those of the proteins. In the presence of solvent perturbants such as polyethylene glycol and ethylene glycol, the bands of the model mixtures are red-shifted to the approximate positions they have in the spectra of the proteins. No additional red shift is seen with solvent perturbation of the proteins, suggesting that the conditions leading to this effect are satisfied within the proteins. A separate effect of solvent perturbation, an increase in amplitude of the positive band, occurs equally in solutions of free aromatic amino acid derivatives and in the proteins. This effect is used to estimate the relative accessibility of the chromophores of fibronectin and its fragment to different perturbants. The possible influence of protein secondary structure on the amplitude of the positive circular dichroic band is discussed.

The fibronectins are closely related glycoproteins of blood plasma and the extracellular matrix that take part in a variety of adhesive functions (see Refs. 1 and 2 for reviews.) These proteins are organized into a series of discrete, independently folded domains (1, 3) that, in some cases, participate in intra- and intermolecular associations (4–6). Owing to the presence of an atypical band (at about 228 nm) in its circular dichroic spectrum, there is no consensus concerning the secondary structure of the protein or that of its constituent domains (7–11).

The understanding of the genesis of the 228 nm CD band of fibronectin would be of central importance in the analysis of the protein's structure and its alterations upon binding various ligands (12). Because the CD spectrum of *N*-acetyltyrosinamide has a strong positive band in the same general region as fibronectin and, also similarly to the glycoprotein, a strong negative band at 183 nm, it has been suggested that the spectrum of fibronectin is dominated by contributions from tyrosine side chains (10, 13). Alterations in the spectral features of fibronectin, particularly in the region of 228 nm, upon denaturation or heparin binding were therefore interpreted in terms of structural modifications in the vicinity of tyrosyl groups (10).

However, a number of additional findings cast doubt on the exclusive attribution of the genesis of the fibronectin 228 nm CD band to tyrosine. The actual wavelength of the *N*-acetyltyrosinamide band, for example, occurs at 225 nm rather than 228 nm (10, 13). Furthermore, whereas the ~230 nm bands of both fibronectin and *N*-acetyltyrosinamide are attenuated at high pH (10), the loss of CD activity of the amino acid derivative is attributable to the reversible ionization of the tyrosine residue, which does not appear to occur in the protein under the same conditions (10). Although structural features of fibronectin may account for some of these discrepancies (8, 11), potential contributions of other amino acid side groups to the protein's spectrum must also be taken into account in any detailed structural analysis (14–17).

In this paper, we report the results of a series of CD studies on human plasma fibronectin and a model mixture containing its constituent aromatic amino acids (*i.e.* the *N*-acetyl ethyl ester derivatives of tyrosine, tryptophan, and phenylalanine) in the same molar ratio as in the protein. A similar analysis has also been performed on the 31-kDa NH_2-terminal heparin-binding fragment of the protein (18), which makes a major contribution to the positive peak at ~228 nm (8), and on its corresponding model mixture of amino acids. Our results indicate that all of the aromatic amino acid side chains contribute to the far-UV CD spectra of fibronectin and its NH_2-terminal fragment. Moreover, solvent perturbation studies suggest that differences between the model mixtures and the proteins with respect to the position of the positive band in the ~230 nm region can be largely accounted for on the basis of increased hydrophobicity of the environments of the aromatic residues in the proteins relative to the model mixtures. We propose that disparities between the magnitudes of the positive CD peaks in the model mixtures and the proteins are likely to result from protein secondary structure with possible contributions from disulfide bonds.

EXPERIMENTAL PROCEDURES

Materials—Human plasma fibronectin was obtained from the New York Blood Center, exchanged into phosphate-buffered saline (0.02 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl) on a Sephadex G-25 column, and used without further purification. The 31-kDa domain of fibronectin was isolated essentially by the procedure of Hayashi and Yamada (18), and its purity was confirmed by electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate. *N*-Acetyl-L-tyrosine ethyl ester, *N*-acetyl-L-tryptophan ethyl ester, *N*-acetyl-L-phenylalanine ethyl ester, polyethylene glycol (M_r = 200), and ethylene glycol were purchased from Sigma. Other reagents were of analytical grade. The standard buffer used throughout was phosphate-buffered saline unless stated otherwise.

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‡ Recipient of a travel award from the Fulbright Program. Permanent address: Dept. of Biochemistry, North Eastern Hill University, Shillong, India.

All experiments were performed at ambient temperature, which was usually 25 °C.

Determination of Concentrations—Fibronectin concentrations were either determined spectrophotometrically at 280 nm using an extinction coefficient of $E = 1280$ cm^2 g^{-1} (19) or by the method of Bradford (20), which was also employed for the estimation of the fragment concentration; bovine serum albumin was used as the standard. Extinction coefficients (cm^2 mol^{-1}) of 1200 at 275 nm, 5500 at 280 nm, and 200 at 257 nm were used for the determination of the molar concentrations of tyrosine, tryptophan, and phenylalanine derivatives, respectively (21). Model mixtures of the aromatic amino acid residues were prepared by mixing the stock solutions of the three amino acids so that their final molar ratio was identical to the ratio in which they are present in fibronectin or its NH_2-terminal fragment. We assumed that 1 mol of fibronectin contains 146, 88, and 82 mol of tyrosine, tryptophan, and phenylalanine, respectively (22). The corresponding values for the fragment were 12, 8, and 3 (23). The molar concentrations of the model mixtures were calculated from their compositions.

Circular Dichroism—Circular dichroic spectra were recorded on a Jasco Model 500C spectropolarimeter equipped with a data processor. The authenticity of the recorded spectra was maintained by keeping the intensity of the signals far below the transmission limit of the instrument, thus ruling out the possibility of any "out-of-light" artifact. In no case was the voltage of the phototube allowed to exceed 500 V at 205 nm. No qualitative differences were observed in the spectra recorded at several concentrations of the proteins, the free amino acid derivatives, or the model mixtures thereof. Other details were the same as described elsewhere (12).

RESULTS

CD Analysis of Fibronectin and Its Constituent Chromophores—Typical CD spectra of fibronectin and its NH_2-terminal fragment are shown in Fig. 1. These spectra are in excellent agreement with earlier reports (7, 11), including the much higher relative intensity of the positive band in the 230 nm region in the fragment than in the intact protein. Not previously reported was our consistent finding that the position of the positive peak in the spectrum of the NH_2-terminal fragment was ~229 nm (*versus* 228 nm for the intact protein), and its maximal negative value was at ~212 nm (*versus* 213 nm for the intact protein).

To assess possible contributions from all three aromatic chromophores toward the formation of the positive CD peak of fibronectin, we studied the CD spectra of tyrosine, tryptophan, and phenylalanine derivatives separately, as well as in combination in the same ratios in which they are present in fibronectin and its 31-kDa fragment. Although the peak positions for tyrosine (λ_{max} = 225 nm), tryptophan (λ_{max} = 224 nm), and phenylalanine (λ_{max} = 214 nm) derivatives are somewhat different from one another, all contribute to a common spectrum that is comparable to that seen with fibronectin. Indeed, a spectrum having a positive peak with its maximal value at ~222 nm results when these chromophores are mixed in a ratio similar to their molar ratios in fibronectin (Fig. 1, *inset*; and Fig. 2, *trace d*).

Because the spectra of the individual amino acid derivatives given in Fig. 2 (*traces a–c*) were recorded from samples whose concentration ratios were equivalent to those in the model mixture for intact fibronectin (Fig. 2, *trace d*), the relative contribution of each chromophore to the 222 nm peak of this mixture can be determined directly from the graphs. We estimate these values to be 44, 32, and 23% for tyrosine, tryptophan, and phenylalanine, respectively. Because phen-

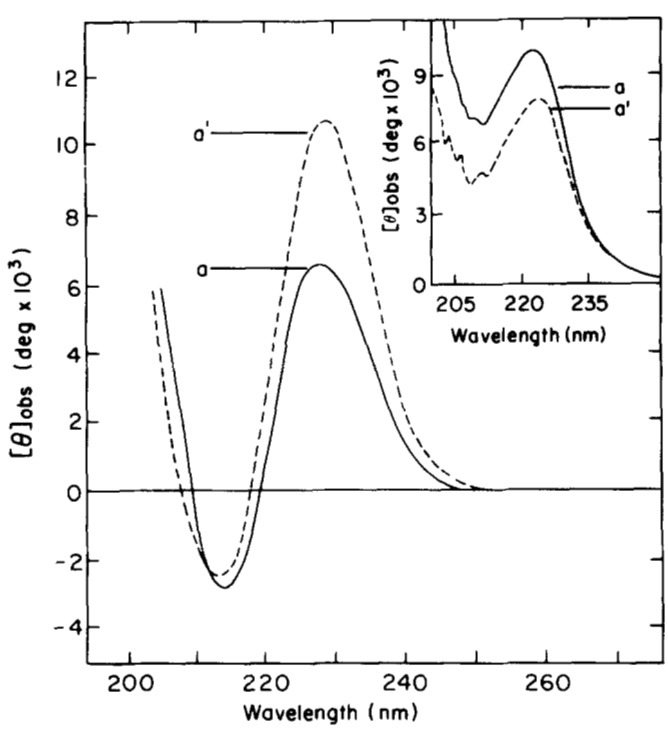

FIG. 1. **Far-ultraviolet CD spectra of human plasma fibronectin (0.04 mg/ml, 8.8 × 10^{-8} M) (*trace a*) and its 31-kDa NH_2 terminal domain (0.05 mg/ml, 1.66 × 10^{-6} M) (*trace a'*) in phosphate-buffered saline at 25 °C.** *Inset*, CD spectra of model mixtures of the *N*-acetyl ethyl esters of phenylalanine, tryptophan, and tyrosine in the ratios in which they are present in fibronectin (*trace a*) and its NH_2-terminal domain (*trace a'*). Assuming molar ratios as given under "Experimental Procedures," the concentrations of the model mixtures for fibronectin and its domain are 1.7 × 10^{-7} and 1.6 × 10^{-6} M, respectively.

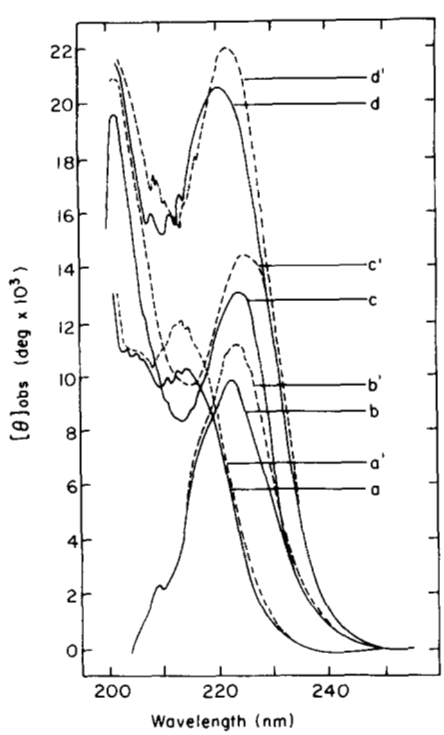

FIG. 2. **Far-ultraviolet CD spectra of *N*-acetyl ethyl esters of phenylalanine (4.2 × 10^{-5} M) (*trace a*), tryptophan (4.4 × 10^{-5} M) (*trace b*), and tyrosine (7.4 × 10^{-5} M) (*trace c*).** These molar values are in the ratio in which the corresponding amino acids are present in human plasma fibronectin (19). A model mixture for fibronectin (3.7 × 10^{-7} M) (*trace d*) was obtained by mixing the three amino acid derivatives in the same proportions. *Traces a'–d'* represent spectra of the preparations in *traces a–d* in the presence of 20% (v/v) polyethylene glycol. Experimental conditions were the same as described for Fig. 1.

ylalanine has its maximum ellipticity at a much shorter wavelength than tyrosine or tryptophan, it plays an important role in determining the position of the peak in any composite of the aromatic amino acids. Indeed, the model mixture for the 31-kDa NH_2-terminal fragment, in which phenylalanine constitutes 13% of the aromatic amino acid derivatives, is red-shifted by more than 1 nm from the model mixture for fibronectin, in which phenylalanine constitutes 26% of such residues (Fig. 1, *inset*).

In order to assess sources of asymmetry in the proteins apart from those contributed by the aromatic chromophores, it is of interest to compare the magnitudes of the peak ellipticity values of fibronectin and its NH_2-terminal domain (Fig. 1) to the peak values for the corresponding mixtures of aromatic amino acid derivatives (Fig. 1, *inset*). We find that the measured peak for intact fibronectin is 19% higher than that calculated for an equimolar concentration of its model mixture. The measured peak for the NH_2-terminal fragment is 40% higher than that calculated for an equimolar concentration of its model mixture.

Solvent Perturbation—Solvent perturbation studies of fibronectin, its NH_2-terminal fragment (Fig. 3), aromatic amino acid derivatives, and mixtures thereof (Fig. 2) were performed using polyethylene glycol, ethylene glycol, and glycerol as perturbants. The concentrations of these nonaqueous substances (10–20%) were large enough to cause measurable shifts in the spectra of chromophoric residues without affecting the conformation of the native protein (24, 25). We found that the amplitude of the positive CD signal of the fibronectin model mixture as well as its constituent amino acid derivatives invariably increased in the presence of these perturbants. The positions of the bands were, moreover, red-shifted by several nanometers (Fig. 2). Solvent perturbation of intact fibronectin and its NH_2-terminal fragment caused similar increases in the amplitudes of the peak signals, but no red shifts (Fig. 3). With polyethylene glycol (M_r = 200) as the perturbant, the extent of increase in the signal intensity was about 20% of the peak value for both the intact protein and fragment, but the majority of the enhancement for the fragment occurred at the lower of the two concentrations of perturbant (Fig. 3*A*). With a smaller perturbant, ethylene glycol, there was a proportionally greater peak increase in the spectrum of the intact protein than in that of the fragment (10 *versus* 6%), but these increases occurred continuously over the concentration range of perturbant for both proteins (Fig. 3*B*). (For brevity, perturbation effects of glycerol, which were qualitatively similar to those of ethylene glycol, are not shown.)

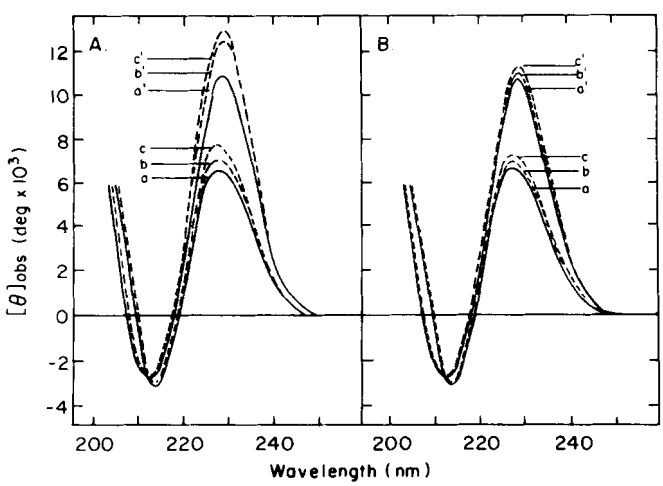

FIG. 3. **Far-ultraviolet CD spectra of fibronectin (*traces a–c*) and its NH_2-terminal domain (*traces a′–c′*) in presence of various concentrations of polyethylene glycol (*A*) and ethylene glycol (*B*).** The concentrations of the two perturbants were: *traces a* and *a′*, 0%; *traces b* and *b′*, 10%; and *traces c* and *c′*, 20%. Other conditions were the same as described for Fig. 1.

DISCUSSION

The strong positive band in the 230 nm region of the CD spectrum of fibronectin is an unusual feature that it shares with only a small number of other proteins (16). On theoretical grounds, strong positive transitions in this region have been calculated to arise from the most probable peptide side chain conformations of L-tyrosine and L-phenylalanine (16); L-tryptophan side chains are also proposed to make similar spectral contributions (16). These predictions have been confirmed by experimental observations in a number of systems (14, 15, 17).

The studies reported here indicate that all three aromatic side chains could make substantial contributions to the positive peaks in the spectra of model mixtures of both intact human plasma fibronectin and the 31-kDa NH_2-terminal fragment thereof. We found that the positions of the positive peaks in the CD spectra of model mixtures are influenced by their phenylalanine content, higher values of which tend to shift the peak to shorter wavelengths. In addition, the polarity of the local environment of the chromophores, as probed by solvent perturbation, also influences the peak position, with solvents of decreasing polarity shifting the peak to longer wavelengths. The slight red shift in the peak of the fragment relative to the intact protein (Fig. 1) may be due to either or both of these effects.

Solvent perturbation has not generally been used in conjunction with CD studies and warrants caution in its interpretation since there are no strong theoretical grounds for expecting analogous wavelength shifts in proteins and their model mixtures. However, our observations are encouraging in that they suggest that adding nonaqueous perturbants to the solvent can in some respects cause the spectra of free chromophores to resemble those of their counterparts in intact proteins. If the conditions promoting such changes already prevail within the interior of a protein, solvent perturbation of the protein would not be expected to induce additional changes in the same direction. In contrast, other effects of these perturbants may arise from conditions that are not, or are only partly, satisfied within the protein interior. These effects would lead to similar changes in the spectra of free chromophores and of accessible chromophores within proteins.

Examination of our solvent perturbation results suggests that we may have observed both types of effects. Solvent perturbation of the individual amino acid derivatives and model mixtures, for example, red shifts their positive CD peaks to wavelengths similar to those of the corresponding peaks in the spectra of fibronectin and its NH_2-terminal fragment (Figs. 1 and 2). Perturbation of the proteins causes no additional red shifts (Fig. 3), suggesting that the conditions for this effect are already satisfied within the proteins. However, the intensification of the spectral peaks resulting from solvent perturbation of the amino acid derivatives and their mixtures (Fig. 2) is similar in magnitude to that in the intact proteins (Fig. 3).

We suggest that perturbation effects of this second type can be particularly useful as probes of protein structure in CD studies. The different dose responses of the positive peaks of the spectra of fibronectin and its NH_2-terminal fragment to the large perturbant, polyethylene glycol (Fig. 3*A*), suggest less obstructed access of this perturbant to the chromophores

in the fragment relative to those in the intact protein, a difference that is not seen when the smaller perturbant, ethylene glycol, is used (Fig. 3*B*). This could be due to long-range interactions within the intact molecule involving the NH_2-terminal domain, a possibility supported by our recent fluorescence polarization studies.[1] The more extensive perturbation by ethylene glycol of intact fibronectin relative to the fragment may represent access of the smaller perturbant to regions of the protein beyond those accessible to polyethylene glycol.

These considerations leave open the question of what determines the absolute magnitude of the positive CD peak in fibronectin and its NH_2-terminal domain, which in each case is greater than would be expected from contributions of aromatic chromophores alone. Nonstandard secondary structure of the extensively disulfide-bonded domains that contribute most strongly to the ~230 nm peak (8) may enhance the positive contribution of the aromatic residues much like β-structure suppresses it (17). However, disulfide bonds themselves also have an inherent optical activity that depends on CSSC bond geometry (26, 27) and, under some circumstances, can contribute to a positive CD peak at ~230 nm (26–28).

We have found that reduction and modification of disulfide bonds in both intact fibronectin and its NH_2-terminal domain diminish the intensity of, but do not eliminate, the ~230 nm spectral peaks (12). Indeed, the spectral peak of the NH_2-terminal fragment, which represents one of the most extensively disulfide-bonded domains of the protein (1–3), retains two-thirds of its optical activity at 229 nm after reduction (12). This suggests that the contribution of disulfide bonds to the positive spectral peak of this domain is less important than that of the aromatic chromophores. This is also likely to be the case for intact fibronectin since 24 of its 28 intramolecular disulfide bonds are contained in structural motifs homologous to the NH_2-terminal domain (2). However, the attenuation of the positive CD peak of intact fibronectin under reduced conditions is proportionally greater than that of the fragment (12). Whereas this is consistent with a greater relative contribution of disulfide bond optical activity to the fibronectin band, this attenuation may also result from acquisition of additional secondary structure by the protein when it is reduced.[1]

In a previous study (12), we demonstrated a variety of changes in the positive CD bands of fibronectin and its NH_2-terminal fragment upon binding various polysaccharide ligands. These ranged from an enhancement of the magnitude of the peak upon binding dextran sulfate to an attenuation of the magnitude and red shift of the peak upon binding heparin (12). The latter change is associated with an adhesive interaction occurring at the cell surface-extracellular matrix interface that has been proposed to play a role in tissue morphogenesis (29, 30). The information obtained in this study may help clarify the specific structural change sustained by fibronectin during this interaction.

[1] M. Y. Kahn, M. Medow, and S. A. Newman, manuscript submitted for publication.

REFERENCES

1. Akiyama, S. K., and Yamada, K. M. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol.* **59,** 1–57
2. Mosher, D. F. (ed) (1988) *Fibronectin*, Academic Press, New York
3. Hynes, R. (1985) *Annu. Rev. Cell Biol.* **1,** 67–90
4. Homandberg, G. A., and Erickson, J. W. (1986) *Biochemistry* **25,** 6917–6925
5. Homandberg, G. A. (1987) *Biopolymers* **26,** 2087–2098
6. Hörmann, H., and Richter, H. (1986) *Biopolymers* **25,** 947–958
7. Koteliansky, V. E., Glukhova, M. A., Benjamin, M. V., Smirnov, V. N., Filimonov, V. V., Zalite, O. M., and Venzyaminov, S. Y. (1981) *Eur. J. Biochem.* **119,** 619–624
8. Odermatt, E., Engel, J., Richter, H., and Hörmann, H. (1982) *J. Mol. Biol.* **159,** 109–123
9. Marković, Z., Lustig, A., Engel, J., Richter, H., and Hörmann, H. (9183) *Hoppe-Seyler's Z. Physiol. Chem.* **364,** 1795–1804
10. Welsh, E. J., Frangou, S. A., Morris, E. R., Rees, D. A., and Chavin, S. I. (1983) *Biopolymers* **22,** 821–831
11. Osterlund, E., Eronen, I., Osterlund, K., and Vuento, M. (1985) *Biochemistry* **24,** 2661–2667
12. Khan, M. Y., Jaikaria, N. S., Frenz, D. A., Villanueva, G., and Newman, S. A. (1988) *J. Biol. Chem.* **263,** 11314–11318
13. Stevens, E. S., Morris, E. R., Charlton, J. A., and Rees, D. A. (1987) *J. Mol. Biol.* **197,** 743–745
14. Shiraki, M. (1969) *Sci. Pap. Coll. Gen. Educ. Univ. Tokyo (Biol. Part)* **19,** 151–173
15. Sears, D. W., and Beychok, S. (1973) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., ed) Part C, pp. 445–593, Academic Press, New York
16. Woody, R. W. (1978) *Biopolymers* **17,** 1451–1467
17. Brahms, S., and Brahms, J. (1980) *J. Mol. Biol.* **138,** 149–178
18. Hayashi, M., and Yamada, K. M. (1983) *J. Biol. Chem.* **258,** 3332–3340
19. Mosesson, M. W., and Umfleet, R. A. (1970) *J. Biol. Chem.* **245,** 5728–5736
20. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
21. Schellman, J. A., and Schellman, C. (1964) in *The Proteins* (Neurath, H., ed) Vol. II, pp. 1–137, Academic Press, New York
22. Mosher, D. F. (1980) *Prog. Hemostasis Thromb.* **5,** 111–151
23. Garcia-Pardo, A., Pearlstein, E., and Frangione, B. (1983) *J. Biol. Chem.* **258,** 12670–12674
24. Herskovits, T. T., and Laskowski, M., Jr. (1962) *J. Biol. Chem.* **237,** 2481–2492
25. Williams, E. J., Herskovits, T. T., and Laskowski, M., Jr. (1965) *J. Biol. Chem.* **240,** 3574–3579
26. Neubert, L. A., and Carmack, M. (1974) *J. Am. Chem. Soc.* **96,** 943–945
27. Kahn, P. C. (1979) *Methods Enzymol.* **61,** 339–378
28. Hider, R. C., Drake, A. F., and Tamiya, N. (1988) *Biopolymers* **27,** 113–122
29. Newman, S. A., Frenz, D. A., Tomasek, J. J., and Rabuzzi, D. D. (1985) *Science* **228,** 885–889
30. Newman, S. A., Frenz, D. A., Hasegawa, E., and Akiyama, S. K. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 4791–4795