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Resonance Raman spectroscopic studies of the interactions between trypsin and a competitive inhibitor

(ligand–enzyme interactions/serine protease/vibrational spectroscopy)

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ABSTRACT **Raman spectroscopy was used to study the interactions between bovine trypsin and a competitive inhibitor. For this purpose, a chromophoric substrate analogue, 4-amidino-4′-dimethylamino azobenzene, was synthesized. This compound competitively inhibits the enzyme with a 1:1 stoichiometry and an inhibition constant K_i of 2.3 μM at pH 6.08 and 15°. Resonance Raman spectra in aqueous solution of free or enzyme-bound inhibitor were analyzed. The main spectral changes observed upon enzyme–inhibitor complex formation were changes in the relative intensities of four bands (1171, 1206, 1315, 1608 cm^{-1}) while no large frequency shifts occurred. The binding of the inhibitor molecule to the enzyme did not induce a twisting of the phenyl groups around the N=N bond. Some modifications of the band widths are interpreted in terms of a restriction of rotational motions in the inhibitor molecule. The possible involvement of specific interactions between trypsin and the benzamidinium ion part of the inhibitor molecule is discussed.**

Many hypotheses have been proposed to explain the efficiency of enzymatic catalysis (1, 2). Several of the explanations which were proposed to account for this efficiency emphasize the role of the binding process. Crystallographic studies can delineate many of the interactions between enzyme and substrate (3–5). Among other methods, resonance Raman spectroscopy represents a powerful method for this purpose, allowing work in aqueous solutions. Some recent studies dealt with protein–ligand interactions: those of a ligand with serum albumin (6), of 2,4-dinitrophenyl haptens with rabbit antibodies (7), and the analysis of spectra of acylchymotrypsins (8).

Resonance Raman spectra can provide some helpful information about the changes of vibrational modes of a substrate molecule when bound to an enzyme and during the other events occurring at the catalytic site. In this work, the binding of a substrate analogue to trypsin was studied by this method. A specific inhibitor, the 4-amidino-4′-dimethylamino azobenzene, was synthesized for this purpose. The consequences of the enzyme–inhibitor complex formation on the resonance Raman spectrum of the inhibitor were analyzed in the region from 1100 cm^{-1} to 1700 cm^{-1}, where the main perturbations were observed.

MATERIALS AND METHODS

Trypsin (Batch no. S 69-1) was a salt-free, crystallized product purchased from Novo Industry. α-Chymotrypsin (Batch no. CD I 2 LX) was a salt-free, three-times-crystallized product purchased from Worthington Biochemical Corp.

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; vib, vibration; stretch, stretching.

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p-Nitrophenyl acetate was an Eastman Kodak product, recrystallized from ethanol. It had mp 76–77° [literature mp 77.3–77.9° (9)]. *N*-α-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester was a Calbiochem product; 4-nitrophenyl-4′-guanidino benzoate came from Nutritional Biochemical Corp. Acetonitrile was purified by the method of Coetzee (10). The buffer used was 2-(*N*-morpholino)ethanesulfonic acid (Mes)·NaOH (11), purchased from Calbiochem.

Preparation of the Inhibitor. Uncorrected melting points are reported, as determined with a Büchi melting point apparatus. The *p*-aminobenzamidine·2 HCl (4 g), synthesized according to Easson and Pyman (12), was dissolved in 65 ml of 2 N HCl at −10°. An aqueous solution of sodium nitrite (1.4 g) was then added dropwise with stirring. After 30 min, the solution was allowed to come to −5°. Then, a solution of *N,N*-dimethylaniline (3.48 g) in 24 ml of 2 N HCl was added and the mixture was allowed to stand at 3° for 1 hr. The resulting precipitate was collected, dissolved in a 1/1 ethanol–water mixture and recrystallized several times in the cold. The product, 4-amidino-4′-dimethylamino azobenzene·2 HCl, (3.5 g of violet needles; 54% yield) had mp 206–207°. (Analysis: calculated for $C_{15}H_{17}N_5 \cdot 2$ HCl: C, 52.93; H, 5.62; N, 20.57; Cl, 20.86. Found: C, 53.11; H, 5.89; N, 20.47; Cl, 20.59).

The derivative 4-amidino-4′-dimethylamino azobenzene·1 HCl was obtained from the dihydrochloride by adding one equivalent of 0.1 N NaOH. The product (a red powder) was crystallized from an ethanol–water mixture. It had mp 255–256°. (Analysis: calculated for $C_{15}H_{17}N_5 \cdot$HCl: C, 59.39; H, 5.97; N, 23.05; Cl, 11.67. Found: C, 59.59; H, 6.18; N, 23.04; Cl, 11.20.)

Kinetic Measurements and Spectroscopic Titrations. The absolute concentration of active sites was determined by measuring the burst of *p*-nitrophenol in the enzymatic hydrolysis of 4-nitrophenyl-4′-guanidino benzoate (13) for trypsin, or *p*-nitrophenyl acetate, as previously described (14), for α-chymotrypsin.

Kinetic measurements were performed at 15 ± 0.05° at pH 6.08 in buffered aqueous solutions (0.025 M Mes·NaOH; 0.1 M NaCl; 0.025 M $CaCl_2$). They were recorded with a Cary 16 spectrophotometer equipped with a thermostated cell compartment and coupled to a Sefram Graphispot recorder. The rates of hydrolysis of *N*-α-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester were obtained by measuring the release of *p*-nitrophenol, using a difference in molar extinction coefficients at 347.5 nm between *p*-nitrophenol and ester of 5050 M^{-1} cm^{-1} (15).

The spectrophotometric determination of the enzyme–inhibitor dissociation constant was carried out on a Cary 118 recording spectrophotometer at 15 ± 0.05°, pH 6.08 (0.025

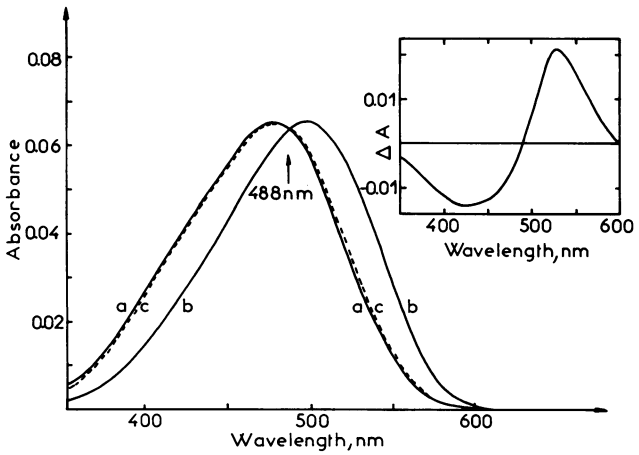


FIG. 1. Effect of trypsin on the visible spectrum of 4-amidino-4'-dimethylamino azobenzene. The spectra were obtained in 0.025 M Mes·NaOH buffer, pH 6.08, containing 0.1 M NaCl, 0.025 M $CaCl_2$, at 15°, at an inhibitor concentration of 2.36 μM. A 1 cm light path was used. Curve (a): 2.36 μM inhibitor; curve (b): 2.36 μM inhibitor in the presence of 48.5 μM trypsin, curve (c): 2.36 μM inhibitor in the presence of 48.5 μM trypsin and 15.7 mM benzamidine. The arrow indicates the exciting laser line. Inset shows the difference spectrum of enzyme plus azobenzene derivative against azobenzene derivative.

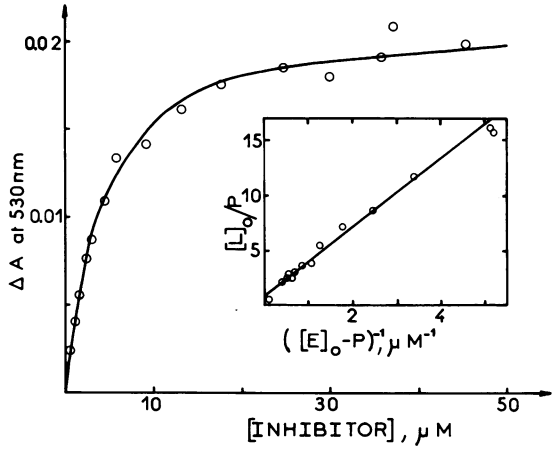


FIG. 2. Spectroscopic titration of trypsin with 4-amidino-4'-dimethylamino azobenzene. The titration was carried out in 0.025 M Mes·NaOH buffer, pH 6.08, containing 0.1 M NaCl, 0.025 M $CaCl_2$, at 15°. The trypsin concentration was 2.42 μM; ΔA is the difference of the absorbance at 530 nm. The line is the theoretical curve calculated for K_i = 2.9 μM and $\Delta\epsilon$ = 8700 M^{-1} cm^{-1}. The inset shows the linear representation of $[L]_0/P$ vs $([E]_0 - P)^{-1}$, according to Wu and Hammes (18); $[L]_0$ and $[E]_0$ are the total ligand and enzyme concentrations, respectively; $P = \Delta A/\Delta\epsilon$ at 530 nm.

M Mes·NaOH; 0.1 M NaCl; 0.025 M $CaCl_2$). Under these conditions, the molar extinction coefficient of the free dye was found equal to 27120 ± 50 M^{-1} cm^{-1} at 480 nm and independent of the concentration between 2.5 μM and 88.5 μM. Experimental data were analyzed with a Wang electronic calculator.

Raman and Infrared Spectra. Raman spectra of the compounds were obtained using laser Raman spectrometers Coderg PH 1 or T 800 with an argon laser (Spectra-Physics 164 AC) emitting the 488 nm or the 514.5 nm lines with 250–350 mW of laser power. A rotating Raman cell was used for solid compounds. For the enzymatic studies, the Raman spectra were obtained with a Coderg PH 1 spectrometer equipped with a rapid scanning system (16) and a data averaging system. The sample cell for the studies of the trypsin–inhibitor complex was previously described (17). It was verified, by measuring the reproducibility of all spectral features as a function of the time of irradiation, that no photochemical decomposition of the inhibitor or photoisomerization about the azo group occurred. Likewise, active site titrations of the enzyme before and after exposure to light have shown the absence of significant inactivation of the protein under our experimental conditions. Infrared spectra were obtained using a Perkin Elmer model 21 spectrometer.

RESULTS

Spectroscopic studies of the enzyme–inhibitor interactions

Addition of 4-amidino-4'-dimethylamino azobenzene to trypsin[¶] produces a red shift of the visible spectrum of the inhibitor (Fig. 1). The difference spectrum shows a maximum at 530 nm, a minimum at 420 nm, and an isosbestic point at 488 nm (Fig. 1, inset). It is possible to reestablish the initial spectrum of the inhibitor by adding benzamidine to the enzyme–dye complex (Fig. 1). A statistical analysis of the binding isotherm (Fig. 2) for the ligand to the enzyme, according to Wu and Hammes (18), leads to an enzyme–inhibitor dissociation constant K_i = 2.9 ± 0.2 μM; the values for the difference molar extinction coefficient at 530 nm and for the number of ligand binding sites are equal to $\Delta\epsilon$ = 8700 ± 160 M^{-1} cm^{-1} and n = 0.80 ± 0.16, respectively.

Inhibition of trypsin by 4-amidino-4'-dimethylamino azobenzene

The effect of 4-amidino-4'-dimethylamino azobenzene on the trypsin-catalyzed hydrolysis of *N*-α-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester is shown in Fig. 3. The Eadie plot demonstrates that the inhibition is competitive. The inhibition constant is found equal to 2.3 ± 0.3 μM at pH 6.08 and 15°, in good agreement with spectroscopic titrations.

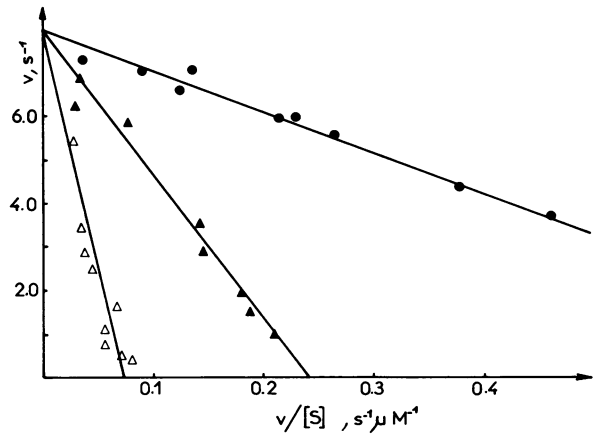


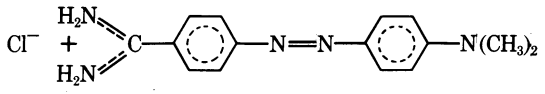
FIG. 3. Inhibition of trypsin by 4-amidino-4'-dimethylamino azobenzene. An Eadie plot of the rate of hydrolysis of *N*-α-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester catalyzed by trypsin is shown. Experimental conditions: pH 6.08 Mes·NaOH buffer, 0.1 M NaCl, 0.025 M $CaCl_2$, at 15°; enzyme concentrations: 0.0145–0.00724 μM; substrate concentrations: 200–5.6 μM; 4-amidino-4'-dimethylamino azobenzene concentrations were: ● zero; ▲ 4.96 μM; △ 24.8 μM.

[¶]We did not observe any variation in the visible spectral features of 4-amidino-4'-dimethylamino azobenzene (16–25 μM) in the presence of α-chymotrypsin (75 μM–1.1 mM) at pH 6.08.

Table 1. Line positions and tentative assignments of Raman and infrared bands of 4-amidino-4'-dimethylamino azobenzene·HCl*

Raman lines (cm^{-1})			Infrared lines (cm^{-1})	
Aqueous ligand	Ligand bound to trypsin	Solid ligand	Solid ligand (KBr pellet)	Tentative assignments
—	—	—	1675 (B)	Amidine group
—	—	1619 (0.5)	—	Ring vib.
1608 (3)	1605 (6)	1600 (3)	1600 (B)	$8^{a,b}$
—	—	—	1543 (s)	Ring vib.
—	—	1525 (<0.5)	1520 (B)	$19^{a,b}$?
1485 (0.5)	—	—	1480 (m)	
1447 (3)	1446 (2.5)	1435 (3)	1445 (m)	N=N stretch vib.
1419 (7.5)	1416 (7)	1414 (5.5)	1420 (B)	coupled with ring vib.
1395 (10)	1393 (10)	1387 (10)	1390 (m)	$19^{a,b}$
1365 (8)	1360 (8.5)	1361 (5.5)	1370 (B)	e_1 (C—N) (dimethylamino group)
—	—	—	1338 (s)	Ring vib. 14
1315 (2.5)	1316 (3.5)	1310 (3)	1315 (m)	and δ (C—H) 3
1266 (0.5)	1266 (0.5)	1262 (1.5)	1260 (m)	—
—	—	1230 (<0.5)	1235 (m)	e_2 (C—N) (azo group)
1206 (1.3)	1209 (0.7)	—	—	δ (C—H) 9^a
1171 (2.5)	1171 (3.5)	1182 (2)	1170 (s)	and e_1 (C—C)
—	—	1160 (0.5)	1158 (s)	(benzamidine group)
1147 (5)	1142 (5)	1133 (6)	1135 (B)	e_1 (C—N) (azo group) and δ(C—H) 9^b

* General formula:



The relative intensity measurements are shown in parentheses. They are only approximate (the highest peak is set arbitrarily equal to 10); s = shoulder, m = medium, B = broad, for the infrared bands; vib. = vibration, stretch = stretching. The line positions and the relative intensity values are the means of five sets of data. The Wilson (19) and Kohlrausch (20) notations are used for the modes of vibration.

Resonance Raman studies

The resonance Raman spectra were recorded from 100 cm^{-1} to 3300 cm^{-1}; Fig. 4 shows the typical resonance Raman spectra of 4-amidino-4'-dimethylamino azobenzene obtained without (a) or with (b) trypsin, from 1100 to 1700 cm^{-1}, where the significant differences appeared. As shown in this figure, the main spectral modifications observed were changes in the relative intensities of four bands (1171; 1206; 1315; 1608 cm^{-1}) while no large frequency shifts occurred. Moreover, a visual comparison of the line widths shows that they are narrower in the case of the trypsin–inhibitor complex than for the free inhibitor in aqueous solution. The line positions for 4-amidino-4'-dimethylamino azobenzene are tabulated in Table 1. By exciting at two different wavelengths (488 nm and 514.5 nm), it was verified that the variations of relative intensities do not result from the red-shift of the electronic band.

DISCUSSION

Kinetic and spectrophotometric studies clearly show that 4-amidino-4'-dimethylamino azobenzene is a competitive inhibitor of trypsin. The values of the inhibition constant K_i are in good agreement whatever the method of measurement may be. This compound proved to be one of the most potent synthetic inhibitors, better than 4-amidinophenylpyruvic acid (21). The modification of the visible spectrum of the inhibitor molecule when bound to trypsin is a classical phenomenon which is observed in the case of several chromophoric inhibitors (22, 23).

The tentative assignments of the Raman bands of 4-amidino-4'-dimethylamino azobenzene were made by comparison with spectra of compounds which constitute parts of the inhibitor molecule, such as benzamidine, *N*-dimethylaniline,

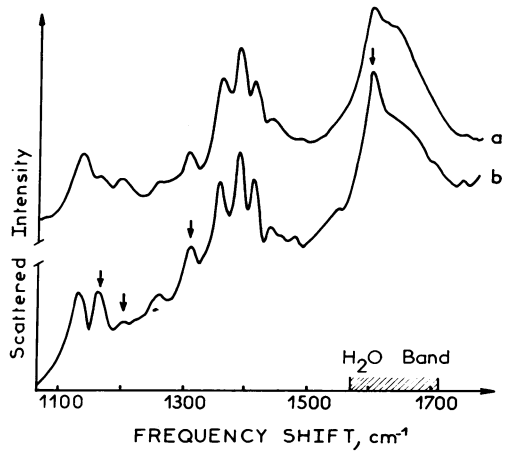


FIG. 4. (a) Resonance Raman spectrum of 3.0 μM 4-amidino-4'-dimethylamino azobenzene in 0.025 M Mes·NaOH buffer, pH 6.08, 0.1 M NaCl, 0.025M $CaCl_2$, at 15°. (b) Resonance Raman spectrum of 3.0 μM 4-amidino-4'-dimethylamino azobenzene bound to 76.2 μM trypsin in the same buffer as (a), at 15°. The arrows indicate the resonance Raman bands whose intensities are modified upon enzyme–ligand complex formation. Instrumental conditions: spectral slit width 4 cm^{-1}, 250 mW of Ar^+ laser power at 488 nm.

N-dimethylamino azobenzene (J. C. Merlin and A. Dupaix, unpublished results), and also with literature data on other related compounds (24–28). In the case of the inhibitor molecule, the observed Raman bands cannot be considered to characterize pure vibrational modes. Indeed, dynamic and kinematic coupling of vibrations are important for this kind of molecule (27, 29).

N=N stretching vibrations (1390, 1450 cm^{-1})

The stretching vibration of the azo group has been studied extensively by different authors (24, 25, 27). In the case of 4-amidino-4′-dimethylamino azobenzene, this vibration appears to be coupled with the vibrational modes 19^a and 19^b of the benzene ring. In the *trans* form, when the benzene rings are coplanar, the N=N stretching vibration is expected in the region 1380–1440 cm^{-1}. When the benzene rings are twisted out of this conformation, a decrease in conjugation throughout the π-electron system leads to an increase in bond order in the N=N linkage. A shift of the N=N stretching band towards higher frequencies and a change in relative intensity of the peak are then expected (7). In the *cis* form, the N=N stretching vibration is expected to occur in the region around 1500 cm^{-1} (7, 27). From these considerations and the observed frequencies for the ligand molecule in this region, it can be concluded, first, that the free molecule in aqueous solution is in the *trans* form with coplanar aromatic rings; second, that, when the inhibitor is bound to trypsin, the N=N linkage is not twisted, as shown by the small changes in frequencies and relative intensities.

C—N stretching vibration (1147, 1365 cm^{-1})

We have to distinguish between the C—N stretching vibration of the aromatic amine and the C—N stretching vibration of the phenyl-N bond of the azo group. The first one is assigned to the peak at 1365 cm^{-1}. A slight shift in frequency is observed (1365→1360 cm^{-1}) when the inhibitor is bound to trypsin, but no change in relative intensity occurs. Thus, this vibrational mode seems to be little perturbed upon binding. The second one is assigned to the peak at 1147 cm^{-1}. A twisting of the phenyl groups out of their aqueous conformation upon enzyme–inhibitor complex formation should provide an increase in the single bond order of the azo C—N bond with a resultant decrease in frequency and a reduction of the resonance Raman intensity (6, 7). Actually, while a small shift of the band towards lower frequencies occurs (1147→1142 cm^{-1}), no change in relative intensity is observed. These observations and the preceding considerations about the N=N linkage lead us to conclude that the binding of the ligand to the enzyme does not produce a twist of the inhibitor molecule.

Benzene ring vibrations (1608, 1485, 1315 cm^{-1})

The change in relative intensities for some in-plane benzene ring vibrations seems to indicate that the electronic distribution of one (or two) benzene ring(s) is perturbed upon binding. Furthermore, the red shift which is observed in the visible spectrum of the ligand molecule reflects an electronic redistribution upon complex formation. Obviously, we cannot distinguish between the vibrations of the two benzene rings. However, it is likely that the binding of the inhibitor molecule occurs by means of the benzamidine part of the molecule, as suggested by our kinetic experiments and by literature data on the binding of benzamidine at the active site of trypsin (30–32).

C—C stretching vibrations (benzamidinium group; 1206, 1171 cm^{-1})

The C—C stretching vibration of the benzamidinium group and the benzene ring vibration 9^a are expected in the region 1206–1171 cm^{-1}. The binding of the inhibitor molecule at the active site of trypsin produces large modifications of the relative intensities of the peaks at 1171 and 1206 cm^{-1}.

CONCLUSIONS

From this work and others (6–8), it appears that resonance Raman spectroscopy is a useful technique for studies of ligand–protein interactions. Generally, resonance Raman spectra are obtained when the exciting light lies in an electronic absorption band. The visible spectrum of 4-amidino-4′-dimethylamino azobenzene is due to conjugation through the π-electron system. Therefore, the enhanced resonance Raman bands can be characterized mainly as vibrational modes of atoms for which the electronic delocalization occurs. Thus, in the resonance Raman spectrum of our ligand, we cannot expect to observe intense bands corresponding to the stretching vibrations of C—N and C—H bonds in the $(CH_3)_2N$ group. Thanks to the electronic delocalization around the amidinium cation of the inhibitor molecule and to vibrational coupling with benzene ring vibrations, it has been possible to observe some changes in peak intensities (i.e., 1171 and 1206 cm^{-1}) when the inhibitor was bound to trypsin. Using x-ray crystallographic data, Krieger *et al.* (33) have shown that benzamidine was bound to trypsin through a salt bridge formed by the amidinium cation of the inhibitor molecule and the negatively charged side chain of Asp 189 in the binding pocket of the enzyme. Furthermore, the benzene ring of this inhibitor was "sandwiched" between the peptide bonds Ser 190-Cys 191 and Trp 215-Gly 216. Such interactions could lead to the observed intensity changes, but other effects, such as hydrophobic environment of the benzamidinium part of the dye, could also account for this phenomenon.

The binding of 4-amidino-4′-dimethylamino azobenzene in the specificity pocket of the enzyme narrows the band widths as compared with free inhibitor. We interpret this change as being due to the freezing of the rotational motions of the inhibitor molecule around the axis with the smallest moment of inertia (34). This interpretation is in agreement with crystallographic results [(33), *vide supra*]. Furthermore, using nuclear magnetic resonance techniques for studying α-chymotrypsin–inhibitor interactions, some authors (35–37) also pointed out that rotational motions of inhibitor molecules were more restricted when bound to the enzyme than in aqueous solution. Studies of spin-labeled trypsin (38, 39) have shown that the mobility of the labels was decreased when bound to the protein.

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1. Koshland, D. E., Jr. & Neet, K. E. (1968) in *Annual Review of Biochemistry*, ed. Boyer, P.D. (Ann. Rev. Inc., Palo Alto, Calif.), Vol. 37, pp. 359–410.
2. Jencks, W. P. & Page, M. I. (1972) *8th F.E.B.S. Meeting, Amsterdam* **29**, 45–58.

3. Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Philipps, D. C. & Varma, V. R. (1967) *Proc. R. Soc. London Ser. B* **167**, 378–388.
4. Blow, D. M. & Steitz, T. A. (1970) in *Annual Review of Biochemistry*, ed. Snell, E. E. (Ann. Rev. Inc., Palo Alto, Calif.), Vol. 39, pp. 63–100.
5. Liljas, A. & Rossmann, M. G. (1974) in *Annual Review of Biochemistry*, ed. Snell, E. E. (Ann. Rev. Inc., Palo Alto, Calif), Vol. 43, pp. 475–507.
6. Carey, P. R., Schneider, H. & Bernstein, H. J. (1972) *Biochem. Biophys. Research. Commun.* **47**, 588–595.
7. Carey, P. R., Froese, A. & Schneider, H. (1973) *Biochemistry* **12**, 2198–2208.
8. Carey, P. R. & Schneider, H. (1974) *Biochem. Biophys. Res. Commun.* **57**, 831–837.
9. Faller, L. & Sturtevant, J. M. (1966) *J. Biol. Chem.* **241**, 4825–4834.
10. Coetzee, J. F. (1967) *Prog. Phys. Org. Chem.* **4**, 45–92.
11. Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S. & Singh, R. M. M. (1966) *Biochemistry* **5**, 467–477.
12. Easson, A. P. T. & Pyman, F. L. (1931) *J. Chem. Soc.* **3**, 2991–3001.
13. Chase, T., Jr. & Shaw, E. (1967) *Biochem. Biophys. Res. Commun.* **29**, 508–514.
14. Dupaix, A., Béchet, J. J. & Roucous, C. (1973) *Biochemistry* **12**, 2559–2566.
15. Seydoux, F., Coutouly, G. & Yon, J. (1971) *Biochemistry* **10**, 2284–2290.
16. Wallart, F. (1970) Thèse de Doctorat d'Etat, Université des Sciences et Techniques de Lille, Lille, France.
17. Da Silva, E. F. & Merlin, J. C. (1974) *Raman News Lett.* **63**, 8–9.
18. Wu, C. W. & Hammes, G. G. (1973) *Biochemistry* **12**, 1400–1406.
19. Wilson, E. B. (1934) *Phys. Rev.* **45**, 706–714.
20. Kohlrausch, K. W. F. (1938) *Der Smekal-Raman Effekt, Ergänzungsband* (J. Springer, Berlin).
21. Markwardt, F., Landmann, H. & Walsmann, P. (1968) *Eur. J. Biochem.* **6**, 502–506.
22. Glazer, A. N. (1965) *Proc. Nat. Acad. Sci. USA* **54**, 171–176.
23. Bernhard, S. A., Lee, B. F. & Tashjian, Z. H. (1966) *J. Mol. Biol.* **18**, 405–420.
24. Hacker, H. H. (1965) *Spectrochim. Acta* **21**, 1989–2004.
25. Gruger, A., Le Calvé, N. & Dizabo, P. (1972) *J. Chim. Phys.* **69**, 291–298.
26. Perrier-Datin, A. & Lebas, J. M. (1972) *J. Chim. Phys.* **69**, 591–600.
27. Gruger, A. & Le Calvé, N. (1972) *J. Chim. Phys.* **69**, 743–750.
28. Perrier-Datin, A., Julien-Laferrière, S. & Lebas, J. M. (1973) *J. Chim. Phys.* **70**, 1684–1691.
29. Vergoten, G. (1973) Thèse de 3e Cycle, Lille, France.
30. Mares-Guia, M., Shaw, E. & Cohen, W. (1967) *J. Biol. Chem.* **242**, 5777–5781.
31. East, E. M. & Trowbridge, C. G. (1968) *Arch. Biochem. Biophys.* **125**, 334–343.
32. Béchet, J. J. & d'Albis, A. (1969) *Biochim. Biophys. Acta* **178**, 561–576.
33. Krieger, M., Kay, L. M. & Stroud, R. M. (1974) *J. Mol. Biol.* **83**, 209–230.
34. Nafie, L. A. & Peticolas, W. L. (1972) *J. Chem. Phys.* **57**, 3145–3155.
35. Marshall, A. G. (1968) *Biochemistry* **7**, 2450–2453.
36. Gerig, J. T. & Reinheimer, J. D. (1970) *J. Am. Chem. Soc.* **92**, 3146–3150.
37. Bittner, E. W. & Gerig, J. T. (1970) *J. Am. Chem. Soc.* **92**, 5001–5003.
38. Morrisett, J. D. & Broomfield, C. A. (1972) *J. Biol. Chem.* **247**, 7224–7231.
39. Berliner, L. J. & Wong, S. S. (1974) *J. Biol. Chem.* **249**, 1668–1677.