Cross-Linking of Ribosomal Proteins by 4-(6-Formyl-3-azidophenoxy)butyrimidate, a Heterobifunctional, Cleavable Cross-Linker†

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ABSTRACT: For the identification of neighbor relationships between proteins in biological systems, 4-(6-formyl-3-azidophenoxy)butyrimidate (FAPB-imidate), a heterobifunctional, cleavable cross-linker was synthesized. The reagent has an imido ester at one end, which is used for the attachment to amino groups of a specific protein whose environment has to be characterized. At the other end, the reagent has both an azido and an aldehyde group. The azido group can be used to cross-link the protein photochemically to a variety of chemical groups of neighboring proteins. The aldehyde group is able to cross-link the protein by reductive alkylation to amino groups of neighboring proteins. In both cases, the cross-linker can be made radioactive with NaB^3H_4. The cross-linked complexes can be split at the band originating from the imidate group by treatment with ammonia. Hereby, the radioactive cross-linker remains covalently attached to the unknown neighboring protein, which can be therefore easily identified. In order to explore the usefulness of FAPB-imidate as a cross-linking agent, the compound was attached to ribosomal protein L7. With this modified L7, the existence of the well-known complex between L7 and ribosomal protein L10 could be demonstrated by the photochemical procedure. By the chemical procedure, the presence of dimers of L7 in solution could be shown.

Several techniques have been used for the elucidation of the ribosome structure, such as immunoelectron microscopy, neutron scattering, and chemical cross-linking (for a review, see Nomura et al., 1974). In particular, the latter technique has been employed for the identification of ribosomal components which are involved in the transient attachment of initiation and elongation factors (Van Duin et al., 1975; Heimark et al., 1976a,b; San José & Kurland, 1976; Fabian, 1976; Acharya et al., 1973). However, the interpretation of these results was often complicated because the cross-linkers were reacted with the whole ribosome which resulted in the formation of many cross-linked complexes. Besides, several of these complexes contained sometimes more than two proteins, making the assignment of neighborship relations ambiguous. In order to circumvent some of these complications, 4-(6-formyl-3-azidophenoxy)butyrimidate (FAPB-imidate),[1] a heterobifunctional, cleavable cross-linker, which can be made radioactive with high specific activities, was synthesized. After attachment of FAPB-imidate via its imidate group to amino groups of one specific protein, cross-linking of this protein to neighboring proteins can be induced in two ways: by reductive alkylation and photochemically. In both cases, treatment of the cross-linked complexes with ammonia results in cleavage of the band, originating from the imidate group.

The usefulness of FAPB-imidate as a cross-linking agent was explored with complexes between proteins from ribosomes from *Escherichia coli*.

Materials and Methods

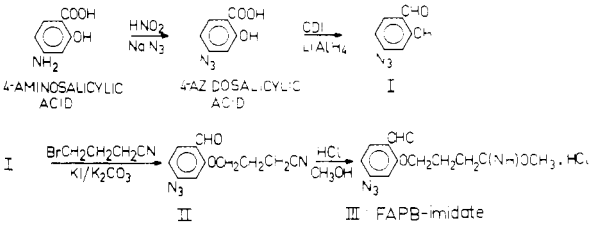
Proteins L7 and L10 were prepared as described (Möller et al., 1972; Hindennach et al., 1971).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis with 12% acrylamide–0.33% *N,N'*-methylenebis(acrylamide) was according to Weber & Osborn (1969).

Thin-layer chromatography was performed on SiO_2-coated aluminum plates (F254; Merck, Darmstadt, West Germany).

Synthetic Procedure. The steps for the synthesis of FAPB-imidate are outlined in Scheme I. The NMR and infrared data of the intermediates were in agreement with their structures.

Scheme I: Outline of the Synthesis of FAPB-imidate[a]



[a] CDI, *N,N'*-carbonyldiimidazole.

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Azidosalicylaldehyde (I). This compound was prepared as described previously (Maassen & Möller, 1978) with the following modification. After reduction, unreacted $LiAlH_4$ was decomposed with some water, after which most of the solvent was removed by rotary evaporation. The residue was suspended in 500 mL of a 1 M sodium phosphate buffer, pH 7.0. This mixture was steam distilled. Compound I is very volatile and solidifies in the condensate as pale yellow crystals: yield, 25%; TLC (ether) R_f = 0.90.

1-(6-Formyl-3-azidophenoxy)-3-cyanopropane (II). I (163 mg, 1 mmol) and 210 mg (1.5 mmol) of 4-bromobutyronitrile were dissolved in 4 mL of dry acetone. To this solution, 70 mg of anhydrous K_2CO_3 and 5 mg of KI were added. This mixture was refluxed for 20 h after which the solvent was removed by rotary evaporation. Ten milliliters of water was added to the residue. This was extracted three times with 10

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[1] Abbreviations used: FAPB-imidate, 4-(6-formyl-3-azidophenoxy)butyrimidate; $NaDodSO_4$ gel electrophoresis, electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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mL of ether. In order to remove unreacted I, the combined ether layers were extracted several times with 0.1 M NaOH until the aqueous layer remained colorless. Compound II was isolated out of the ether layer via its bisulfite-addition product. This was filtered off, washed with ether, and decomposed with 1 M H_2SO_4. The precipitate of II was collected by filtration, washed with water, and dried: yield, 60%; TLC (ether) R_f^{II} = 0.57. The absorption spectrum of II, recorded in acetonitrile–water (9:1), is shown in Figure 1, panel A. The molar extinction coefficients at 325 and 292 nm are 10 500 and 15 900 M^{-1} cm^{-1}, respectively.

1-(6-Formyl-3-azidophenoxy)butyrimidate (FAPB-imidate, III). All steps were performed under anhydrous conditions. II (300 mg) was dissolved in a mixture of 3 mL of methanol and 9 mL of ether. This solution was cooled to −78 °C and was saturated with HCl gas. The mixture was kept overnight at −30 °C. Subsequently, 60 mL of ether was added. At this point, the solution became slightly opaque. The mixture was kept for a few hours at −30 °C until the insoluble material was precipitated. The clear supernatant was poured into 500 mL of ether. This mixture was kept for a few hours at −30 °C, resulting in the precipitation of III as yellow crystals. These were filtered off and dried in vacuo. The yield of III was 45%.

The compound is readily soluble in water in which it is not stable. It is much more convenient to use solutions of III in dry dimethyl sulfoxide. These are stable for at least 2 h at room temperature: NMR (CH_3SOCH_3-d_6) δ 10.2 (singlet, C*H*O), 6.8 and 7.65 (multiplet, aromatic H's), 4.15 (triplet, O–CH_2–), 4.00 (singlet, –OCH_3), 2.82 (triplet, –CH_2–C(=NH)–O$^-$), 2.05 (multiplet, –$CH_2CH_2CH_2$–); IR (KBr) 2800 (C=NH·HCl), 2120 (–N_3), 1680 cm^{-1} (aromatic aldehyde). The absorption spectrum of III is identical with the spectrum of II.

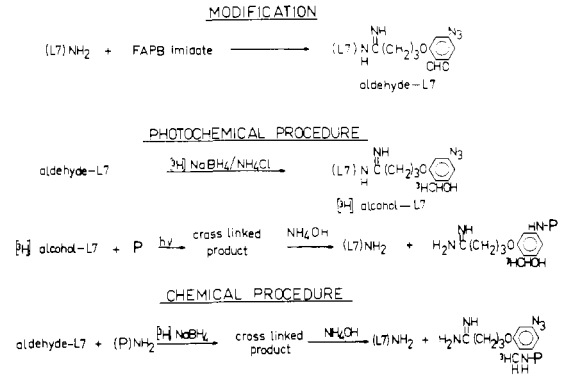
Coupling of FAPB-imidate to Ribosomal Protein L7. To a 1 mg/mL solution of L7 in a 0.1 M sodium borate–HCl buffer, pH 8.6, was added a solution of FAPB-imidate in dry dimethyl sulfoxide at a concentration of 4 mg/mL. Eighty micrograms of FAPB-imidate was added for each milligram of protein. The mixture was kept for 1 h at 0 °C after which the procedure was repeated. Then, the reaction mixture was made 60 mM in NH_4Cl and the protein was isolated by gel filtration on Sephadex G-25, equilibrated with a buffer containing 50 mM sodium borate–HCl, pH 8.6, 60 mM NH_4Cl, 3 mM 2-mercaptoethanol. This modified L7 will be henceforth referred to as "aldehyde-L7". Assuming a molar extinction coefficient at 325 nm of 10 500 M^{-1} cm^{-1}, the number of FAPB-imidate molecules bound to the protein can easily be determined. In this case, 1–1.3 mol of FAPB-imidate was incorporated. The UV spectrum of "aldehyde-L7" is shown in Figure 2, panel B.

Cross-Linking. These procedures are outlined in Scheme II.

Photochemical Cross-Linking of L7 to L10. (a) *Tritiation of Aldehyde-L7.* To a 1 mg/mL solution of aldehyde-L7 in 50 mM sodium borate–HCl, pH 8.6, 60 mM NH_4Cl, and 3 mM 2-mercaptoethanol, a 0.2 M solution of NaB^3H_4 (specific activity 5 Ci/mmol) in 2 M NaOH was added with vigorous stirring. Five microliters of borohydride solution was used for each milliliter of protein solution. The reduction was complete within a few seconds as indicated by the disappearance of the absorption band at 325 nm (Figure 2, panel C).

(b) *Cross-Linking.* Ten micrograms of ^{3}H-labeled alcohol-L7 and 10 μg of L10 were dissolved in 250 μL of buffer (20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 60 mM NH_4Cl, and 6 mM 2-mercaptoethanol). The mixture was irradiated for 15 min at 0 °C with a Philips SP500W super-high-pressure mercury lamp, equipped with a glass filter cutting off wavelengths below 320 nm (Maassen & Möller, 1977).

Scheme II: Outline of the Cross-Linking Procedures[a]



[a] P, neighboring protein. During the reduction of aldehyde-L7 by $NaBH_4$ in the presence of NH_4Cl, part of the aldehyde is converted into a benzylamine derivative.

(c) *Ammonolysis.* The irradiation mixture was made 6 M in NH_4OH and 1 M in acetic acid and incubated for 16 h at 30 °C (Barritault et al., 1975). Ammonia was removed by dialysis and the proteins were separated by $NaDodSO_4$ gel electrophoresis. After staining by Coomassie Brilliant Blue R, the gels were sliced. Slices were dissolved in 30% H_2O_2 by heating at 60 °C for 16 h and counted in Instagel (Packard).

Chemical Cross-Linking of L7 to Itself. (a) *Cross-Linking.* Aldehyde-L7 was dissolved at a concentration of 1 mg/mL in 50 mM sodium borate–HCl, pH 8.6, 20 mM NH_4Cl, and 3 mM 2-mercaptoethanol. The presence of NH_4Cl is required in order to suppress formation of oligomers of L7 (cf. Discussion). To 200 μL of this solution, cooled to 0 °C, 5 μL of a 0.2 M NaB^3H_4 solution in 2 M NaOH was added with vigorous stirring. Stirring was continued for another 5 min at 0 °C, after which samples were taken for analysis on $NaDodSO_4$ polyacrylamide gels. In order to isolate the dimer, the proteins in the reaction mixture were separated on a Sephacryl S-200 column (0.7 × 70 cm), equilibrated with 50 mM sodium borate, pH 8.6, and 0.1% $NaDodSO_4$.

(b) *Ammonolysis.* This was performed as described under photochemical cross-linking.

Results

The procedure for cross-linking of proteins, chemically and photochemically, by FAPB-imidate is outlined in Scheme II. Based on measurements on a Dreiding model of FAPB-imidate, the maximal spans are 1.0 and 0.8 nm in photochemical and chemical cross-linking, respectively.

The modification of amino groups of proteins via the imidate group of FAPB-imidate proceeds smoothly at a pH of 8.6 and a temperature of 0 °C. This modification takes place with retention of the positive charge of the amino group (Hand & Jencks, 1962). The modification reaction is terminated by addition of an excess of NH_4Cl, after which the protein is isolated by gel filtration.

The number of FAPB-imidate groups incorporated is easily quantitated from the absorbance at 325 nm. At this wavelength, the 6-formyl-4-azidophenoxy chromophore possesses an absorption maximum with a molar extinction coefficient of 10 500 M^{-1} cm^{-1} (Figure 1, panel A). It is assumed that incorporation into proteins does not significantly influence this value. As an example, in Figure 2 (panels A and B) are shown

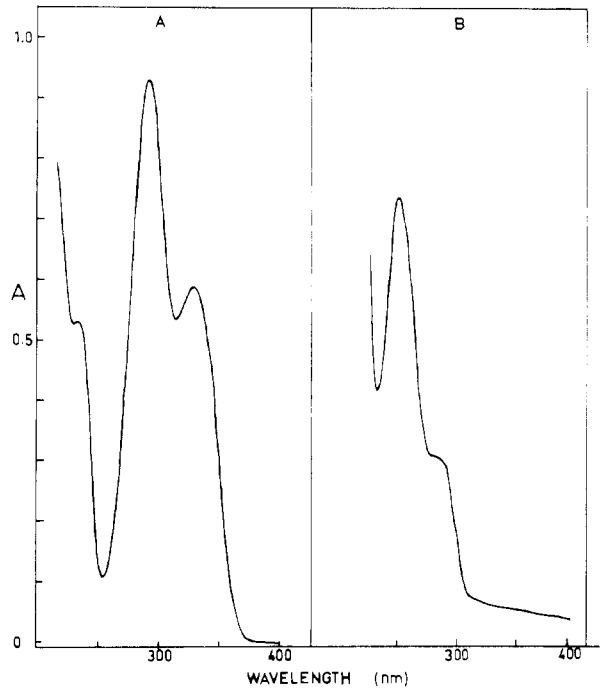


FIGURE 1: Absorption spectra of FAPB-imidate (panel A) and of FAPB-imidate, reduced with $NaBH_4$ (panel B). Solvent was CH_3CN–H_2O (9:1). Concentration of FAPB-imidate was 60 μM.

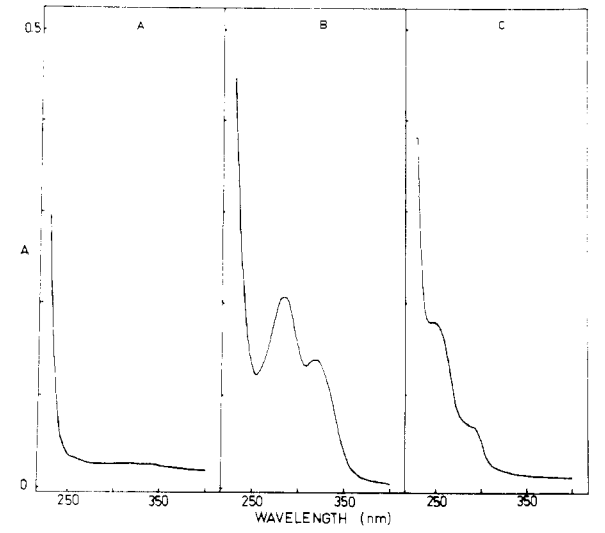


FIGURE 2: Absorption spectra of ribosomal protein L7 (panel A), of aldehyde-L7 (panel B), and of alcohol-L7 (panel C). Protein concentrations were 0.2 mg/mL. Solvent was 50 mM sodium borate–HCl, pH 8.6.

the absorption spectra of unmodified protein L7 and modified L7, containing 1.2 mol of FAPB-imidate per mol of protein. In the case of photochemical cross-linking (Scheme II), prior to the actual cross-linking, the modified protein is made radioactive by reduction of the aldehyde group with NaB^3H_4. This reduction is performed in the presence of a large excess of ammonium ions (60 mM) in order to prevent chemical cross-linking of the protein by reductive alkylation of the aromatic aldehyde group with lysine residues (Means & Feeney, 1968). The course of the reduction can be monitored by the disappearance of the absorption band at 325 nm. In Figure 1, panel C, is shown the absorption spectrum of alcohol-L7. For comparison, the spectrum of FAPB-imidate, reduced with $NaBH_4$, is given in Figure 1, panel B.

The use of NaB^3H_4 with a specific activity of 5 Ci/mmol results in specific activities of 2000–4000 dpm/pmol for each

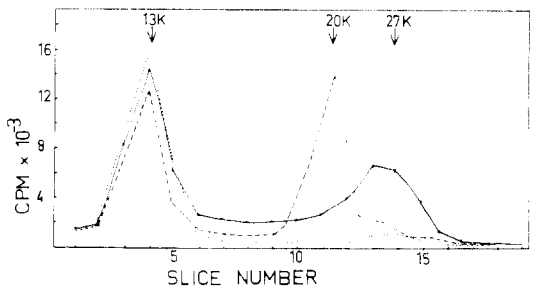


FIGURE 3: $NaDodSO_4$ gel electrophoresis of the reaction product obtained after photochemical cross-linking and ammonolysis. (---) Radioactivity profile after irradiation of ^3H-labeled alcohol-L7 and L10. (—) Radioactivity profile after irradiation of ^3H-labeled alcohol-L7 alone, followed by another irradiation in the presence of L10. (···) Radioactivity profile of unirradiated ^3H-labeled alcohol-L7.

aldehyde group reduced. It is essential that the mixture is stirred well during the addition of NaB^3H_4. Otherwise, because of the fast hydrolysis of NaB^3H_4 with respect to nonradioactive $NaBH_4$, a decrease of the specific activity of NaB^3H_4 takes places during the course of the reduction, which results in lower specific activities of alcohol-L7. In order to cross-link photochemically the ribosomal proteins L7 (mol wt 13400) and L10 (mol wt 19000), these proteins form a stable complex (Petterson et al., 1976); ^3H-labeled alcohol-L7 was mixed with L10. Cross-linking was induced by irradiation with ultraviolet light with wavelengths above 320 nm.

The yield of covalently cross-linked complexes was too low for detection on $NaDodSO_4$–polyacrylamide gels. Ammonolysis of the irradiated protein mixture led, however, to the appearance of radioactivity at the position of L10 on the gel, as shown in Figure 3. Furthermore, it can be seen in this figure that prephotolysis of ^3H-labeled alcohol L7, before adding L10, does not result in the appearance of radioactivity at the position of L10. In this case, only some radioactivity is present at the position of dimeric L7, due to an incomplete ammonolysis of this dimer.

The procedure for chemical cross-linking is outlined in Scheme II. As a model system we have chosen ribosomal protein L7, which, in solution, is present as a noncovalent dimer (Möller et al., 1972). Reduction of aldehyde-L7 to alcohol-L7 by $NaBH_4$ in the absence of ammonium ions gave covalently bound dimers and multimers of L7. This cross-linking took place by a process of reductive alkylation (Means & Feeney, 1968) because it could be largely suppressed by performing the reduction in the presence of compounds bearing amino groups (Tris; NH_4Cl) at concentration above 60 mM. If the concentrations of these compounds were lower (20 mM) mainly dimers of L7 would be formed in yields of about 50% (Figure 4, gel 2).

In order to explore whether dimeric L7, prepared by the chemical cross-linking procedure, could be converted by ammonolysis into monomeric L7, dimeric L7 was purified by gel filtration on Sephacryl S-200 (Figure 4, gel 3). This purified L7 dimer was treated with ammonia–acetic acid after which it was reanalyzed on $NaDodSO_4$–polyacrylamide gels. As shown in Figure 4, gel 4, part of the dimer was converted into monomeric L7. If ^3H-labeled L7 dimer was used, which was prepared by carrying out the cross-linking with NaB^3H_4, ammonolysis resulted in the formation of radioactive monomeric L7 (not shown).

Discussion

We have explored the usefulness of FAPB-imidate as a reagent for the identification of neighbor relations between proteins by means of transferring a small, radioactive molecule

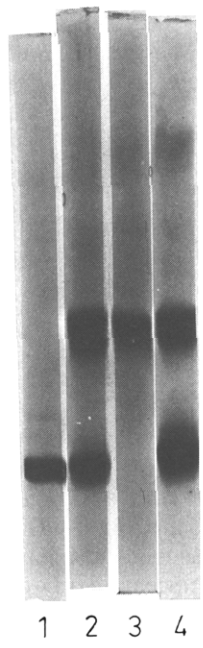


FIGURE 4: $NaDodSO_4$–polyacrylamide gels of (1) aldehyde-L7; (2) aldehyde-L7, chemically cross-linked in the presence of 20 mM NH_4Cl; (3) purified dimeric L7, isolated from the protein mixture shown in gel 2 by gel filtration on Sephacryl S-200; (4) ammonolysis product of purified dimeric L7.

from one specific protein to another, unknown, neighboring protein (Scheme II). As model systems, the complexes between ribosomal proteins L7 and L10 and the L7 dimer were used. Attachment of FAPB-imidate to L7 proceeds smoothly under relatively mild conditions (0 °C; pH 8.6). Under these conditions, only one FAPB-imidate molecule becomes incorporated. Analysis of the tryptic peptide map of modified L7 indicated that attachment of FAPB-imidate had taken place at Lys-29 or Lys-51; both are located in a hydrophobic part of the molecule (results not shown). This specific modification may be the result of the hydrophobic nature of FAPB-imidate.

During the attachment of FAPB-imidate to the protein via the imidate group, the aromatic aldehyde moiety of FAPB-imidate may form a Schiff base with amino groups of the proteins in a reversible manner. Upon removal of excess of cross-linker by gel filtration or dialysis, this reaction is easily reversed, leaving behind that part of the cross-linker which is attached to the protein via the imidate group (cf. Williams & Jacobs, 1968). That all of the cross-linker is indeed bound to L7 via the imidate group is indicated by the fact that exhaustive dialysis of aldehyde-L7 against 1 M acetic acid, a condition under which Schiff bases are completely hydrolyzed, does not lead to a decrease in the derivatization of aldehyde-L7.

After the modification step, cross-linking can be accomplished in two ways: photochemically and chemically. In both cases, the aromatic part of the cross-linking molecule becomes tritiated if NaB^3H_4 is used.

After cross-linking, the complexes are split by ammonolysis. This cleavage at the position originating from the imidate group requires rather harsh conditions (6 M NH_4OH–1 M acetic acid for 16 h at 30 °C). However, ribosomal proteins do not seem to be affected significantly by this treatment (Barritault et al., 1975). The yield of the cleavage reaction is about 70%, as estimated from Figure 4, gel 4.

It is shown in this paper that both the photochemical procedure (L7 to L10) and the chemical method (L7 to L7) are suitable for the identification of neighbor relationships. The low yield of the photochemical cross-linking seems to be inherent in cross-linking via aromatic azides (Bayley & Knowles, 1977).

Yields of cross-linking by the procedure of reductive alkylation were much higher. In the presence of 20 mM Tris or NH_4Cl, this method gave a 50% yield of covalently bound L7 dimers, without significant formation of multimers. In the absence of NH_4Cl or Tris, multimers were also formed. This indicates that, in the presence of compounds with amino groups (NH_4Cl, Tris) in concentrations of 20 mM, very specifically the cross-linking is confined to the noncovalent L7 dimers. This specific cross-linking could be largely suppressed if the concentrations of amino group containing compounds were higher than 60 mM. Though in our case the yields with chemical cross-linking were much higher than with photochemical cross-linking, the former process requires that the aromatic aldehyde group and amino groups of neighboring proteins are in close vicinity to each other. In contrast, cross-linking via aromatic azides does not demand specific structural requirements.

Although cross-linking and ammonolysis are far from quantitative, the procedure described here is quite sensitive because the cross-linker can be made radioactive with specific activities in the order of several thousand dpm/pmol. It should be emphasized that FAPB-imidate is only applicable in those cases where a specific protein can be removed from a biological system and, after modification with the cross-linker, can be added back. This condition can be frequently fulfilled in in vitro studies of ribosomes. Presently, this compound is used for the characterization of the attachment sites for the elongation factors G and Tu on the bacterial ribosome.

Acknowledgments

I thank Dr. Wim Möller for his many helpful and stimulating discussions. I am indebted to Dr. P. I. Schrier for his gift of protein L10, to J. D. J. Bakker for preparing protein L7, and to Dr. R. J. Deleys for critical reading of the manuscript.

References

Acharya, A. S., Moore, P. B., & Richards, F. M. (1973) *Biochemistry 12*, 3108.

Barritault, D., Expert-Bezancon, A., Milet, M., & Hayes, D. H. (1975) *FEBS Lett. 50*, 114.

Bayley, H., & Knowles, J. R. (1977) *Methods Enzymol. 46*, 111.

Fabian, U. (1976) *FEBS Lett. 71*, 256.

Hand, E. S., & Jencks, W. P. (1962) *J. Am. Chem. Soc. 84*, 3505.

Heimark, R. L., Kahan, L., Johnston, K., Hershey, J. W. B., & Traut, R. R. (1976a) *J. Mol. Biol. 105*, 219.

Heimark, R. L., Hershey, J. W. B., & Traut, R. R. (1976b) *J. Biol. Chem. 251*, 7779.

Hindennach, I., Kaltschmidt, E., & Wittmann, H. G. (1971) *Eur. J. Biochem. 23*, 12.

Maassen, J. A., & Möller, W. (1977) *Methods Enzymol. 46*, 649.

Maassen, J. A., & Möller, W. (1978) *J. Biol. Chem. 253*, 2777.

Means, G. E., & Feeney, R. E. (1968) *Biochemistry 7*, 2192.

Möller, W., Groene, A., Amons, R., & Terhorst, R. (1972) *Eur. J. Biochem. 25*, 5.

Nomura, M, Tissiéres, A., & Lengyel, P. (1974) *Ribosomes*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Petterson, I., Hardy, S. J. S., & Liljas, A. (1976) *FEBS Lett. 64*, 135.
San-José, C., & Kurland, C. G. (1976) *FEBS Lett. 71*, 133.
Van Duin, J., Kurland, C. G., Dondon, J., & Grunberg-Manago, M. (1975) *FEBS Lett. 59*, 287.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Williams, J. N., & Jacobs, R. M. (1968) *Biochim. Biophys. Acta 154*, 323.

Interaction of α-Chymotrypsin with the Fluorescent Probe 1-Anilinonaphthalene-8-sulfonate in Solution†

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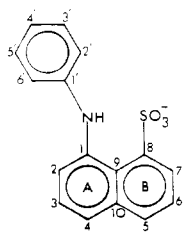
ABSTRACT: The binding of the fluorescence probe 1-anilinonaphthalene-8-sulfonate (Ans) to α-chymotrypsin (α-CHT) at pH 3.6 is accompanied by a dramatic enhancement of Ans fluorescence and a shift of the emission maximum to shorter wavelengths. Our study reveals that one Ans molecule binds to α-CHT at a site different from either the active site of α-CHT or the 2-*p*-toluidinylnaphthalene-6-sulfonate binding site. The binding constant of Ans is about the same (10^4 M^{-1}) at pH 3.6 and 6.4. Nanosecond fluorescence depolarization data indicate that Ans is rigidly bound to α-CHT. The fluorescence enhancement due to binding of Ans to α-CHT at low pH could be due to binding either to a hydrophobic site or to a site where local dipoles do not relax during the excited-state lifetime of Ans. As the pH is increased, fluorescence intensity of the Ans–α-CHT complex decreases appreciably, and the emission maximum shifts to longer wavelengths. The fluorescence decay curves exhibit a corresponding sensitivity to pH. The pH effect on the fluorescence of Ans–α-CHT can be interpreted in terms of a pH-dependent equilibrium between α-CHT conformers differing in the degree of mobility of polar residues and water molecules at the Ans binding site or structural changes in the Ans binding site.

1-Anilinonaphthalene-8-sulfonate (Ans) (I) has been used



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extensively as an extrinsic fluorescent probe of biological macromolecules and membranes (Brand & Gohlke, 1972). In constrast to the weak green fluorescence of Ans in aqueous solutions, an intense blue fluorescence is exhibited when Ans binds in proteins and membranes. Fluorescence spectra, fluorescence polarization, quantum yields, and decay times of Ans are sensitive to the microenvironment of the Ans probe. Such sensitivity has made Ans a popular probe to monitor structural changes in proteins and membranes. The dependence of the emission properties of Ans on the environment derives from an increase in its permanent dipole moment as a result of excitation and the subsequent relaxation of the dipoles of the environment to achieve an equilibrium excited-state configuration. This leads to a red shift of the fluorescence emission maximum and a decrease in the fluorescence yield in polar media. Thus, the polarity of the environment can be measured if the relaxation of the medium occurs during the lifetime of the excited state. However, if the dipoles of the environment are restricted in motion as in a protein molecule or in a viscous medium, relaxation may not be complete during the lifetime of the excited state, giving rise to an intense fluorescence at shorter wavelengths similar to Ans in nonpolar media. It is clear, therefore, that caution must be exercised in deriving conclusions regarding the characteristics of protein sites from fluorescent-probe studies.

Since Ans is used extensively to monitor conformation changes in proteins and to give an indication of the degree of polarity of specific protein sites, it becomes of vital importance to correlate the structural features of the Ans binding site with changes in the fluorescence properties of Ans as a result of binding or other factors such as pH change. This prompted us to a detailed study of the Ans interaction with α-chymotrypsin (α-CHT) using fluorescence and X-ray crystallographic techniques. In this paper, we report fluorescence studies of Ans interaction with α-CHT in solutions under various conditions. In the next paper, X-ray crystallographic results and fluorescence properties of Ans–α-CHT crystals under various conditions are presented. The structural details of the Ans binding site are discussed, and a mechanism of fluorescence enhancement at low pH (3.6) and of fluorescence quenching at higher pH (6.6) is presented. Our results clearly suggest that fluorescence spectral shifts and intensity changes due to binding of a fluorescent probe to a protein molecule can be misleading with regard to the polarity of the binding site.

Experimental Section

Materials. α-CHT, three-times recrystallized and with an activity of 54–61 units/mg, and tosylated α-CHT were obtained from Worthington Biochemical Corp. and were used without further purification. We obtained β-phenylpropionic acid (βPP) from Fluka, *N*-formyltryptophan from International Chemical and Nuclear Corp., proflavin (3,6-di-

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0006-2960/79/0418-1292$01.00/0