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A New Fluorescent Probe for Protein and Nucleoprotein Conformation. Binding of 7-(*p*-Methoxybenzylamino)-4-nitrobenzoxadiazole to Bovine Trypsinogen and Bacterial Ribosomes*

R. A. Kenner† and A. A. Aboderin‡

ABSTRACT: A new fluorescent compound, whose fluorescence is strongly dependent on the polarity of the solvent, is described as a probe of hydrophobic areas on proteins and nucleoprotein particles. The synthesis and fluorescence of 7-(*p*-methoxybenzylamino)-4-nitrobenz-2-oxa-1,3-diazole (MBD) are described. MBD belongs to a class of compounds which are strongly fluorescent in solvents of low polarity and essentially nonfluorescent in water. When MBD is bound to a protein molecule, the fluorescence is that of the fluorophore in a low polarity solvent, suggesting that MBD is bound to a hydrophobic area on the macromolecule. To test the usefulness, specificity, and sensitivity of this new fluorescent probe, MBD was applied to bacterial ribosomes and to bovine trypsinogen and trypsin. MBD binds tightly to 50S and 70S ribosomal subunits but not to the 30S subunit. A dissociation constant (K_f) of 5–9 μM^{-1} for the 50S and 70S subunits was measured. Binding of MBD to ribosomes is interpreted in terms of a unique hydrophobic pocket created between protein molecules as a result of protein–nucleic acid interaction. MBD differentially binds the zymogen, trypsinogen, and the enzyme, trypsin. The K_f for binding to trypsinogen at pH 5.0 is 50 μM^{-1}, but only $\sim$0.3 mM for trypsin. The decrease in binding of the fluorophore has been followed during the activation of trypsinogen to trypsin and found to follow the kinetics of autoactivation. Changes in binding that occur during this transformation of zymogen to enzyme are described and interpreted in terms of a configurational difference between the inactive and active forms of the protein.

While in recent years X-ray crystallography has provided the structure of several enzyme molecules in the crystalline state, our knowledge of the dynamic aspects of the conformation of proteins in solution has depended on less direct experimental approaches. The spectral properties, ionization states, and chemical reactivity of amino acid side chains have long been used as valuable indicators of side-chain interactions, but often these methods are too limited in sensitivity

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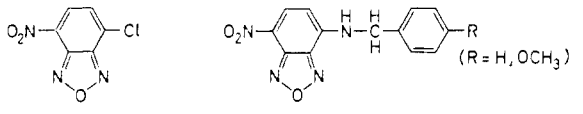

7-chloro-4-nitrobenz-oxadiazole (NBD-Cl)

7-benzylamino-4-nitrobenz-2-oxa-1,3-diazole (BBD)

7-(*p*-methoxybenzylamino)-4-nitrobenz-2-oxa-1,3-diazole (MBD)

FIGURE 1: Structure of MBD, BBD, and NBD-Cl.

to detect small configurational changes. Fluorescent compounds which are highly sensitive to microenvironmental polarity changes can be covalently or noncovalently conjugated to proteins as extrinsic "reporter groups" (Burr and Koshland, 1964) to signal information about local environmental changes. Such fluorescent conjugates of macromolecules have been extensively investigated by Weber and coworkers (Weber, 1952; Weber and Laurence, 1954; Weber and Young, 1964) and more recently by Stryer (1965, 1968), Laurence (1966), Brand *et al.* (1967), McClure and Edelman (1966, 1967a,b), Parker *et al.* (1967), Chen and Kernohan (1967), Haugland and Stryer (1967), and Kenner and Neurath (1971). However, the range of conjugates for probing the hydrophobic areas of proteins has been mainly limited to derivatives of 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride[1]), 1-anilinonaphthalene-8-sulfonate (ANS), and 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS).

Recently Ghosh and Whitehouse (1968) described a new reagent, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD chloride) (Figure 1), which reacts with amino groups to form stable, highly fluorescent compounds. We describe in this communication the use of two closely related derivatives of NBD-Cl, 7-benzylamino-4-nitrobenz-2-oxa-1,3-diazole (BBD) and 7-(*p*-methoxybenzylamino)-4-nitrobenz-2-oxa-1,3-diazole (MBD) (Figure 1), as sensitive fluorescent reporter groups of nonpolar areas and conformational changes in proteins and nucleoprotein particles. MBD and BBD have been used to follow the conformational change associated with the autoactivation of bovine trypsinogen. Also binding of MBD to the 50S ribosomal subunit of *Escherichia coli* is described and interpreted in terms of a unique hydrophobic pocket resulting from protein–nucleic acid interaction.

Experimental Procedure

Materials. All solvents were reagent or spectroscopic grade. Lyophilized bovine trypsin (lot 6009421) was obtained from C. F. Boehringer and Soehne GmbH. Once crystallized bovine trypsinogen (lot 93630) was obtained from Fluka AG. *p*-Nitrophenyl-*p'*-guanidinobenzoate hydrochloride (NPGB) was synthesized and used as an active site titrant for trypsin as described by Chase and Shaw (1968).

Ribosomes were prepared from *E. coli* MRE600 or a K12 strain (RFS524). *E. coli* cells were grown to late log phase by the method described by Moore *et al.* (1968) and homogenized with glass beads (Chamberlin and Berg, 1962), buffered at pH 7.4 with 20 mM Tris-HCl (Trizma base, Sigma Co.)–10 mM $MgOAc_2$–100 mM NH_4Cl. Glass beads and cell debris were removed by low-speed centrifugation, and washed 70S ribosomes were prepared by layering the supernatant over approximately one-third volume of 20 mM Tris-HCl (pH 7.4)–20 mM $MgOAc_2$–0.5 M NH_4Cl–0.9 M sucrose (RNase free, Schwarz Bioresearch, Inc.) and then pelleted using a Beckman rotor 60Ti at 50,000 rpm for 12 hr; 70S ribosomal particles were dissociated into 50S and 30S subunits by dialysis against 0.1 M KCl–1 mM $MgOAc_2$–10 mM Tris-HCl, pH 7.4. Large quantities of pure 50S and pure 30S subunits were prepared by zonal ultracentrifugation in a titanium B-XV rotor as described by Eikenberry *et al.* (1970).

TABLE I: Elemental Analyses of MBD and BBD.[a]

Element	MBD (mp 174–177°; mol wt 300) Expected (%)	MBD Found (%)	BBD (mp 207–208.5°; mol wt 270) Expected (%)	BBD Found (%)
C	55.95	55.88	57.78	57.74
H	4.00	4.21	3.73	3.86
O[b]	21.31	21.38	17.76	17.79
N	18.65	18.53	20.73	20.61

[a] Analyses were performed by A. Bernhardt, 5251 Elbach über Engelskirchen, West Germany. [b] By difference.

Synthesis of MBD and BBD. NBD-Cl was synthesized according to Boulton *et al.* (1966a,b). BBD and MBD (Figure 1) were prepared by treating 4 mmoles of NBD-Cl with 4.7 mmoles of benzylamine or 4.3 mmoles of *p*-methoxybenzylamine, each in 80 ml of ethyl acetate at room temperature. After 2 hr of reaction, 15 ml of water was added to remove excess amine. The ethyl acetate layer was dried with anhydrous $MgSO_4$ and then evaporated to dryness. Dark green crystals of BBD (yield 74%) or reddish brown crystals of MBD (yield 75%) appeared. Both BBD and MBD were recrystallized twice from absolute ethanol. After recrystallization from ethanol, both MBD and BBD gave single spots on several systems of thin layer chromatography. Elemental analyses gave the expected composition for both compounds (Table I).

Spectral Measurements. All fluorescence data uncorrected for instrument response were collected on an Hitachi Perkin-Elmer MPF-2A spectrofluorometer. A "Spectro" 210 Turner spectrophotometer–spectrofluorometer was utilized for all absorption and corrected fluorescence excitation and emission spectra. As a result of the double monochromator design of the Turner 210 spectrophotometer, possible fluorescence artefacts in absorption spectra of highly fluorescent compounds are eliminated. Quantum efficiencies were evaluated using quinine bisulfate as a standard. The details of the method were described earlier (Kenner, 1971). All fluorescence determinations made on the Turner were on samples having a total absorption at the exciting wavelength of less than 0.01.

Measurements of Binding. McClure and Edelman (1967) developed the theory for the noncovalent interaction of small fluorophore molecules with proteins. The simplest case they considered is applicable to the binding of MBD and BBD to trypsinogen and bacterial ribosomes. This case invokes an interaction between an enzyme (E) and a fluorophore (F) to

[1] The following abbreviations are used: dansyl chloride, 1-dimethylaminonaphthalene-5-sulfonyl chloride; ANS, 1-anilinonaphthalene-8-sulfonate; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; NBD chloride, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; BBD, 7-benzylamino-4-nitrobenz-2-oxa-1,3-diazole; MBD, 7-(*p*-methoxybenzylamino)-4-nitrobenz-2-oxa-1,3-diazole; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate hydrochloride.

TABLE II: Absorption Properties of MBD and BBD in Ethanol–Water Mixtures.

% Ethanol (v/v)	MBD Wavelength (nm)	MBD ϵ (M^{-1} cm^{-1})	BBD Wavelength (nm)	BBD ϵ (M^{-1} cm^{-1})
3.2	267	7,000	263	5,000
3.2	345	8,000	343	8,000
3.2	480	26,200	478	24,600
100	280	6,300	264	4,200
100	330	8,000	330	8,000
100	462	20,400	462	19,700
Isosbestic points:	273[a]	6,400	277	3,600
	381	700	381	1,100
	458[a]	21,000[a]	456	19,500

[a] Approximate position and value of isosbestic point.

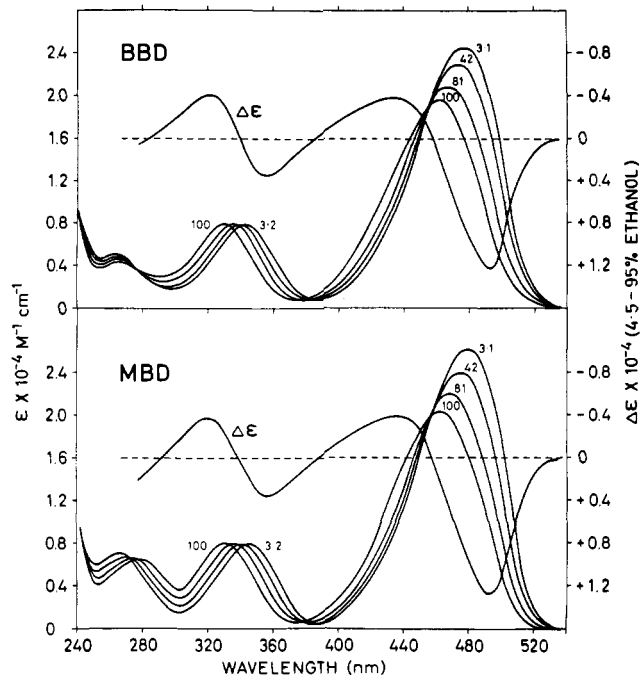

FIGURE 2: Absorption spectra of MBD and BBD in ethanol–water mixtures. The percentages of ethanol in water (v/v) are indicated on the curves. $\Delta\epsilon$ is the difference of ϵ for each compound in 4.5% ethanol and in 95% ethanol.

form a binary complex (C_f) with a dissociation constant, K_f, as described by

$$E + F \rightleftharpoons^{K_f} C_f$$

If only the binary complex, C_f, shows significant fluorescence, the observed intensity of fluorescence, I, is simply related to C_f by a proportionality constant. Thus the concentration of the binary complex is directly measurable. McClure and Edelman derived eq 1 to describe this case.[2] I_{max} represents the

$$I = I_{max} - K_f \frac{I}{F_0} \quad (1)$$

maximal fluorescence of the fluorophore–protein complex and F_0 the total fluorophore concentration (*i.e.*, $F_0 = F + C_f$). A plot of I *vs.* I/F_0 will yield a straight line[3] with a slope of K_f.

Although eq 1 assumes only the formation of a binary complex, *i.e.*, binding of only one fluorophore molecule to one enzyme molecule, it is unlikely that the precision of this fluorescence method is sufficient to distinguish between binding by the enzyme (or nucleoprotein particle) of more than one fluorophore molecule with dissociation constants of the same order of magnitude.

Measurements of binding of MBD and BBD to trypsinogen and trypsin were made at protein concentrations of approximately 0.1 mM. Binding experiments were usually conducted at pH 5.0 utilizing a potassium acetate (0.05 M) buffer containing 0.1 M KCl and 0.025 M $CaCl_2$. In the case of measurements at pH 8.0, the Tris-HCl (0.05 M) buffer contained 0.1 M KCl, 0.025 M $CaCl_2$, and 0.1 mM benzamidine to prevent activation of trypsinogen which could arise from the presence of small amounts of trypsin in the trypsinogen preparation. All fluorescence measurements on trypsin and trypsinogen were made at 25°.

Measurements of binding of MBD and BBD to *E. coli* ribosomes were made utilizing essentially the same conditions Nomura and coworkers used for reconstituting biologically active 30S ribosomes from proteins and 16S RNA (Traub and Nomura, 1969; Mizushima and Nomura, 1970). All measurements were made at 25° at either pH 8.0 or 6.5. The buffer at pH 8.0 contained 0.01 M Tris-HCl, 0.02 M $MgCl_2$, and 0.2 M KCl. The pH 6.5 buffer contained 0.05 M potassium phosphate, 0.02 M $MgCl_2$, and 0.2 M KCl. Binding measurements on 50S and 70S subunits were made at 8 mg/ml and on 30S subunits at 6 mg/ml.

MBD and BBD solutions for titrating proteins and ribosomes were made up in 50% ethanol (v/v) to 50–70 μM. In a typical binding experiment, a 1-cm cuvet containing 2 ml of protein or ribosome solution was titrated with 10–20 μl aliquots of fluorescent probe. The fluorescence was recorded at each addition of fluorophore and the appropriate "blanks" were subtracted for each measurement. The excitation monochromator of the spectrofluorometer was set at either 340 nm or 458 nm with a 10-nm band pass. The emission monochromator was usually set at 530 nm with a band pass of 10 nm. To avoid a significant drift in the response of the Hitachi fluorometer, it was necessary to make each complete set of measurements within about 20 min.

Results

Absorption Spectra of MBD and BBD. The absorption spectra of MBD and BBD in ethanol–water mixtures are are shown in Figure 2 and summarized in Table II. The only

[2] The fluorophore, F, is significantly fluorescent in water (see Figure 5). Thus the total fluorescence intensity, I, of the unbound fluorophore, F, and the binary complex, C_f, cannot be directly substituted into eq 1. If the concentration of F $\gg$ E, as is the case for all the measurements reported here, the difference between the fluorescence intensities in the presence and absence of E will give ΔI, the fluorescence intensity of the binary complex. A plot of ΔI *vs.* $\Delta I/F_0$ will then yield a straight line of slope K_f.

[3] Deviation from a straight line may occur when only a small excess of fluorophore over protein is used. For a discussion of this problem, see the derivation of eq 1 by McClure and Edelman (1967a).

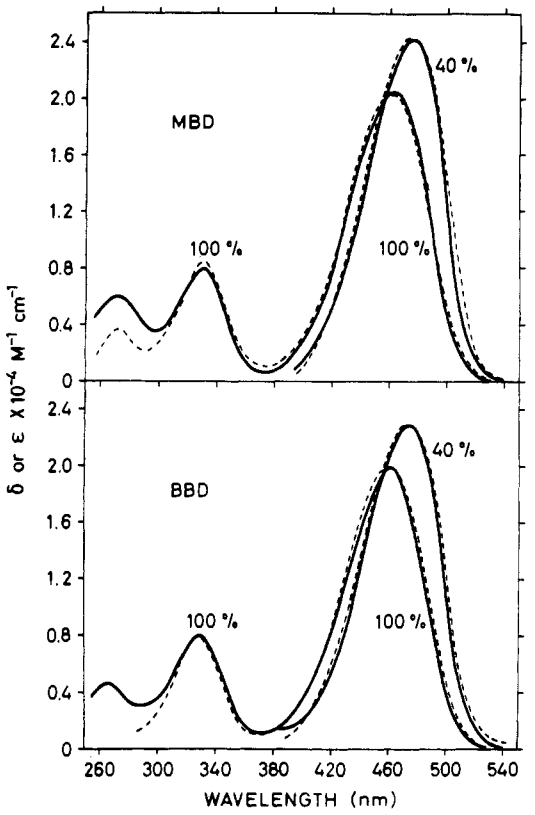

FIGURE 3: Excitation spectra of MBD and BBD. The excitation spectra were measured in either 40 or 100% ethanol. The absorption spectra (solid curves) were measured using a resolution of 2.5-nm band pass. δ is the corrected excitation extinction spectra (hatched curves) normalized to ϵ at 460 nm for 100% ethanol or 475 nm for 40% ethanol. The excitation spectra were measured using a band pass of 2.5 nm on the excitation monochromator and 10 nm on the emission monochromator set at 550 nm.

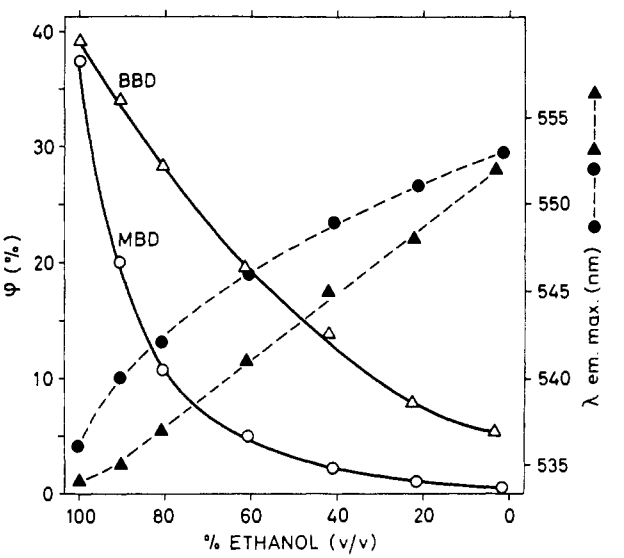

FIGURE 4: The variation of quantum yield, ϕ (%), and the wavelength of maximum fluorescence emission, $\lambda_{em\ max}$, of MBD and BBD in ethanol–water mixtures. ϕ (%) (BBD, Δ; MBD, O) and $\lambda_{em\ max}$ (corresponding hatched curves) were determined using band passes of 10 nm for both emission and excitation monochromators. Excitation in each case was at 335 nm.

major difference between the two chromophores is the greater molar absorptivity, ϵ, of MBD in the 460–490-nm absorption band. The molar absorptivity of MBD in 3.2% ethanol (v/v) is about 6% greater at its absorption maximum at 480 nm than BBD at its corresponding maximum at 478 nm. Absorptivity in the 330–350-nm band appears to be identical for both compounds at all concentrations of ethanol (ϵ = 8000 M^{-1} cm^{-1}). There is a characteristic shift of this absorption band from about 350 nm in water to about 330 nm in ethanol without a change in magnitude of the absorptivity. A corresponding shift also occurs for both compounds in the longer wavelength absorption band from a maximum in water of approximately 480 nm to 462 nm in ethanol, but in this band the absorptivity changes by about 20%. Similar results are obtained for the spectra of NBD-glycine (Aboderin and Kenner, unpublished).

In Figure 2, difference spectra ($\Delta\epsilon$) are also shown representing differences in absorptivity of the chromophores in water and in ethanol solutions. These difference spectra were measured on a Cary 16S double-beam spectrophotometer by placing a chromophore solution containing 95% ethanol in the reference beam and 4.5% ethanol in the analyzing beam. Approximately symmetrical positive and negative peaks of equal absolute $\Delta\epsilon$ (~7000 M^{-1} cm^{-1} for both MBD and BBD) are observed in the 290–380-nm region. A large positive $\Delta\epsilon$ (~12,000 M^{-1} cm^{-1}) occurs for both chromophores at 492 nm, representing the strong dependence of both the absorptivity and absorption maximum of the chromophores on solvent polarity.

Fluorescent Properties of MBD and BBD. Both MBD and BBD fluoresce strongly in the range of 535–555 nm when excited with light in the 330-nm or 460-nm regions. Figure 3 illustrates that the quantum yield of fluorescence is constant at all wavelengths of light from 320 to 520 nm. In this figure the corrected excitation spectra, δ, at two concentrations of ethanol are compared to the absorption spectra, ϵ, for both fluorophores. The large Stokes' shift of ~200 nm for excitation in the 330-nm band is especially useful for measurements made in the presence of macromolecular systems, *e.g.*, ribosomes, which readily scatter light.

Table III lists the quantum yields, ϕ, and wavelengths of maximum fluorescence emission, λ_{max}, for MBD and BBD in several alcohols of different polarity and in water. The quantum yields are high with λ_{max} at shorter wavelengths (~535 nm) in alcohols of lower polarity (ϕ = 0.35 to 0.45), but quite low with λ_{max} at 552–553 nm in water.

The potential usefulness of MBD and BBD as fluorescent probes of hydrophobic microenvironments is illustrated by the results shown in Figure 4. The changes in quantum efficiencies and wavelengths of maximum emission with changes in solvent composition are reminiscent of the dependence of TNS

TABLE III: Quantum Yields of MBD and BBD.

	MBD		BBD	
Solvent	λ_{max} (nm)	ϕ	λ_{max} (nm)	ϕ
1-Butanol	534	0.442	532	0.427
1-Propanol	534	0.448	532	0.411
Ethanol	535	0.358	532	0.379
Methanol	538	0.215	536	0.360
Water (3% ethanol)	553	0.005	552	0.055

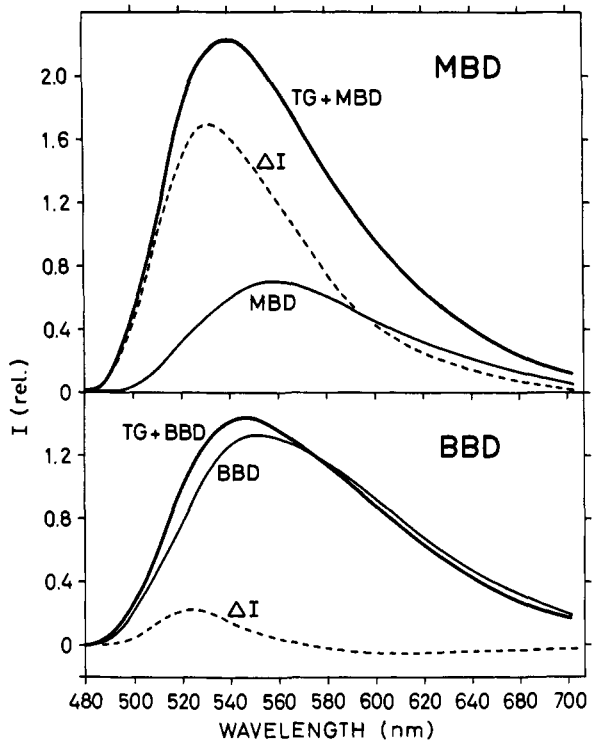

FIGURE 5: Fluorescence spectra of trypsinogen–fluorophore complexes. The solid curves are the fluorescence of MBD (6 μM) or BBD (5 μM) alone and trypsinogen (0.1 mM) added to MBD (TG + MBD) or BBD (TG + BBD). The hatched curve, ΔI, in each case represents the point-by-point difference in relative fluorescence intensity, I_{rel}. The fluorescence spectra are corrected to quanta per unit bandwidth. The instrument settings were the same as those given in Figure 4.

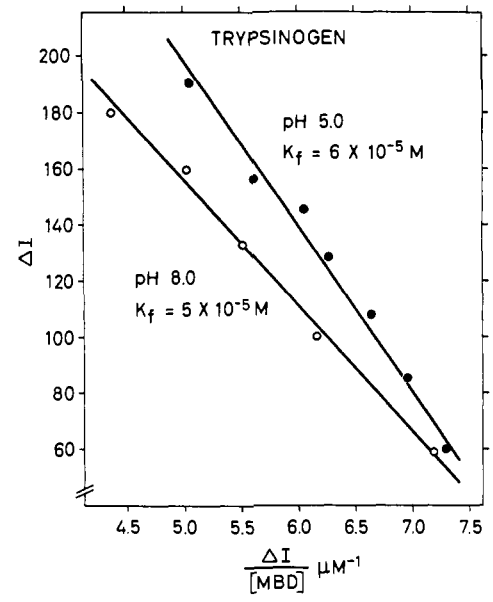

FIGURE 6: Fluorescence titration of trypsinogen with MBD. The titration data are plotted according to eq 1. The trypsinogen concentration was 0.14 mM. Fluorescence, ΔI, was measured using 10-nm band passes on both the excitation and emission monochromators. Excitation was at 458 nm and emission at 530 nm. For other details, consult the text.

(McClure and Edelman, 1966) and of danylaminotyrosine derivatives (Kenner, 1971) on solvent polarity: as the solvent is changed from ethanol to water, the quantum efficiencies drop from near 40% for both MBD and BBD to 0.5% for MBD and 5.5% for BBD. At the same time, the wavelength of maximum fluorescence emission increases from approximately 535 to 553 nm. MBD demonstrates more sensitivity toward the polarity of the environment than BBD, *i.e.*, the quantum efficiency for MBD decreases with increased solvent polarity much more rapidly than BBD and finally reaches a substantially lower level (Figure 4).

Binding of MBD and BBD to Trypsinogen and Trypsin. The corrected fluorescence emission spectra shown in Figure 5 illustrate the method of measurement as well as some of the characteristics of the binding of MBD and BBD to trypsinogen at pH 5.0. The two solid curves in each case represent either the fluorescence of free fluorophore (MBD or BBD) or free fluorophore plus a trypsinogen–fluorophore complex (TG + MBD or TG + BBD). The hatched curves (ΔI) represent the point-by-point difference in intensities of free fluorophore and the corresponding trypsinogen–fluorophore complex. Binding of either MBD or BBD to trypsinogen results in a greater fluorescence intensity of the fluorophore and a corresponding decrease in wavelength of maximum emission. As suggested by the properties of MBD and BBD in ethanol–water mixtures (Figure 4), the direction of change in the two parameters measured, ϕ and λ_{max}, indicates binding of the fluorophores to nonpolar areas on the proteins.

By measuring the magnitude of ΔI in a titration series and plotting the results according to eq 1, a dissociation constant, K_f, of the binding of MBD to trypsinogen was determined at pH 5.0 and 8.0 to be 60 μM^{-1} and 50 μM^{-1}, respectively (Figure 6). Similarly, the binding of BBD to trypsinogen was measured at pH 5.0. In this case, K_f is estimated to be 35 μM^{-1} (Table IV). The enzyme trypsin also binds MBD at pH 5.0, but much less tightly than the zymogen. K_f for the trypsin–MBD complex is estimated to be 0.3 mM^{-1} (Table IV).

The differential binding of MBD to trypsinogen and trypsin was more carefully investigated. The change in MBD binding was followed as a function of active sites generated during activation of trypsinogen. The experiment was performed by autoactivating trypsinogen at 0°, pH 8.0 (0.01 M Tris-HCl, 0.025 M $CaCl_2$), with a ratio of added trypsin to trypsinogen of 1:15. At time intervals between 0 and 120 min, aliquots of the

TABLE IV: Dissociation Constants for MBD and BBD Binding.

Material	pH	K_f (μM) MBD	K_f (μM) BBD
50S ribosomes	8.0	7	ND[a]
50S ribosomes	6.5	5	ND
50S ribosomes (0.5 mM $MgCl_2$)	6.5	5	
70S ribosomes	8.0	5	ND
70S ribosomes	6.5	9	ND
70S ribosomes (K12 strain)	6.5	15	
Trypsin	5.0	300	ND
Trypsinogen	5.0	60	35
Trypsinogen[b]	8.0	50	

[a] ND, very weak binding (>mM). [b] 10 mM benzamidine added as trypsin inhibitor.

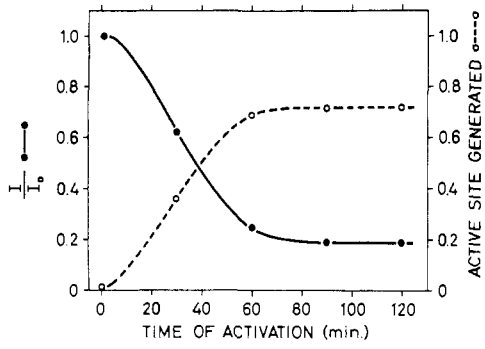

FIGURE 7: Changes in binding of MBD during the activation of trypsinogen. The hatched curves represent the generation of active sites during the autoactivation of trypsinogen. (For details on the activation and measurement of active sites, consult the text.) The solid curve, I/I_0, represents the decrease in binding of MBD as activation of trypsinogen progresses. I_0 is the fluorescence of the trypsinogen-MBD complex (trypsinogen, 0.08 mM; MBD, 30 μM), and I is the fluorescence as the activation progresses.

activation mixture were removed for determination of the active sites generated as judged by titration with NPGB and fluorescence intensity at pH 5.0 at a fixed MBD concentration. The results are shown in Figure 7. As the transformation of the zymogen to the enzyme progresses, *i.e.*, generation of active sites, there is an approximately proportional apparent decrease in the binding of MBD.

Binding of MBD and BBD to Other Proteins. Of the additional proteins investigated, none bound MBD or BBD. Neither fluorophore binds to bovine chymotrypsinogen A or α-chymotrypsin at pH 5.0. Hen egg white lysozyme was investigated at both pH 5.0 and 8.0, but there was absence of fluorophore binding at both pH values.

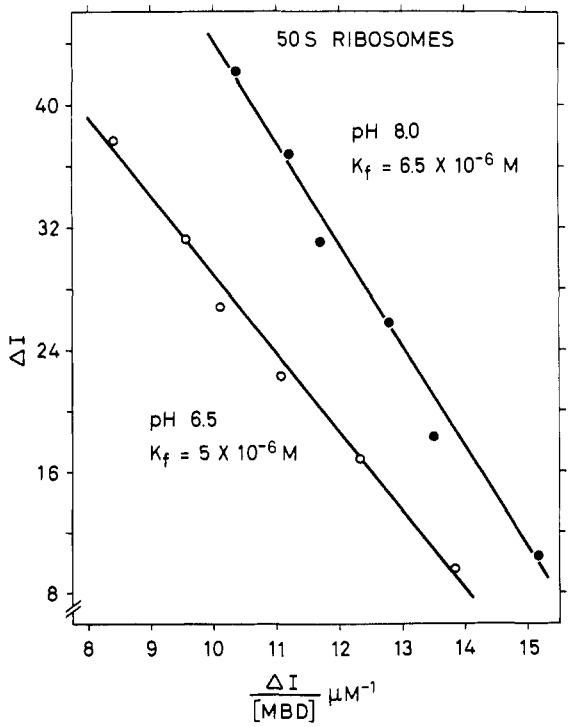

FIGURE 8: Fluorescence titration of 50S ribosomal subunits with MBD. The ribosome concentration was 8 mg/ml. All other details are the same as those given in Figure 6.

Binding of MBD to Ribosomes. Figure 8 shows data plotted according to eq 1 for the binding of MBD by *E. coli* (MRE-600) 50S ribosomal subunits. The dissociation constant in the presence of 20 mM Mg^{2+} and at pH 8.0 and 6.5 are 7 μM^{-1} and 5 μM^{-1}, respectively. K_f for 50S subunits appears not to be affected by lowering the magnesium concentration to 0.5 mM, a level at which 70S subunits dissociate into 30S and 50S subunits. However, a complete mixture of proteins derived from LiCl extraction of 5S and 23S RNA from 50S subunits (Leboy *et al.*, 1964) does not bind MBD. Furthermore, neither 23S RNA from 50S subunits nor 16S RNA from 30S subunits is capable of binding MBD.

Ribosomes (70 S) from *E. coli* (MRE600) also bind MBD. At pH 6.5 and 8.0, K_f is 9 μM^{-1} and 5 μM^{-1}, respectively (Table IV). Ribosomes (70 S) derived from a K12 strain of *E. coli* bind MBD (K_f = 15 μM^{-1}), but the binding appears to be less strong than in ribosomes from the MRE600 strain. The difference between the ribosomes from the two strains, however, is not sufficient to suggest different conformations.

Even at high concentrations of MBD and 30S ribosomal subunits, no significant binding is observed at either pH 6.5 or 8.0. Contrary to the results with MBD, no significant binding of BBD with either the 70S or the 50S subunit can be measured at all concentrations of fluorophore.

Discussion

MBD as a Hydrophobic[4] *Probe.* The data presented above reveal the sensitivity of benzylamino derivatives of NBD-Cl as fluorescent probes of hydrophobic regions in proteins and nucleoprotein particles.[5] In particular, the *p*-methoxybenzylamino derivative, MBD, demonstrates the potential of this series of fluorescent compounds. (1) MBD has a favorably high quantum yield in environments of low polarity (ϕ = 0.36 in ethanol) and a very low quantum yield in water (ϕ = 0.005) (Table II). (2) Associated with the large change in quantum efficiency, there is a large change in the wavelength of maximum fluorescence emission when the solvent polarity is changed (Table II). (3) Two distinct absorption bands, one with a modest solvent-independent molar absorptivity of 8000 M^{-1} cm^{-1} and another with a very strong solvent-dependent molar absorptivity of 20,400 to 26,200 M^{-1} cm^{-1}, are available for fluorescence excitation. (4) The quantum yield is independent of the absorption band used for excitation (Figure 3). (5) Use of the 335-nm absorption band for excitation provides a large Stokes' shift of ~200 nm for fluorescence, a necessary requirement for measurement of low levels of fluorescence in the presence of large macromolecular systems which readily scatter excitation light.

[4] Ainsworth and Flanagan (1969) point out that the terms "hydrophobic" and "nonpolar" are not synonymous for describing binding sites on proteins. Since insufficient data are available to make a distinction between hydrophobic and nonpolar binding sites on either ribosomes or trypsinogen, we do not intend to imply, by using the term "hydrophobic," that the binding sites are best described as hydrophobic rather than nonpolar.

[5] The two benzylamino derivatives we discuss here are uncharged, but other fluorescent charged or uncharged derivatives of NBD-Cl, designed for specific macromolecules, can easily be synthesized from the NBD chloride reagent and essentially any compound containing a free amino group (Ghosh and Whitehouse, 1968), including α- and ϵ-amino groups in proteins. It should, however, be pointed out that aromatic amines readily react with NBD-Cl but result in compounds of low fluorescence yield (Ghosh and Whitehouse, 1968; R. A. Kenner, unpublished). For example, the NBD derivative of ethyl *N*-acetyl-3-aminotyrosinate, analogous to the dansylaminotyrosine derivatives described by Kenner (1971), is essentially nonfluorescent.

With both MBD and BBD the absorption maximum and the absorptivity change substantially in the 460-nm region when the solvent polarity is changed. The change in absorptivity ($\Delta\epsilon$) is $\sim$12,000 M^{-1} cm^{-1} at 492 nm when the solvent is changed from ethanol to water, but, in the 335-nm absorption band, changes in solvent composition serve only to shift the absorption maximum without changing the absorptivity. Thus it is to be expected that both absorption and excitation spectra will be shifted from longer to shorter wavelengths in the 335- and 460-nm bands and the absorptivity decreased in the 460-nm region when MBD binds to a hydrophobic region of a macromolecule (Figures 2 and 3). At the same time, the fluorescence emission maximum should decrease (Figure 5 and Table III). With both trypsinogen and ribosomes, binding of MBD (and BBD in the case of trypsinogen) results in shifts of the absorption and excitation spectra to shorter wavelengths in the 335-nm region and a decrease in the wavelength of maximum fluorescence emission. No change in either the absorptivity or the absorption and excitation maxima is detected in the 460-nm band. One interpretation of this surprising result is that the conformation of the fluorophore when bound to trypsinogen or ribosomes is the same as that in water. A space-filling model of MBD demonstrates that the compound is free to take on either an extended configuration as illustrated in Figure 1 or one which places the *p*-methoxybenzyl group in a nonzero dihedral angle with the nitrobenzoxadiazole group. Presumably, one conformation is thermodynamically favored in ethanol and another in water, giving rise to large absorption changes when the solvent is changed (Figure 2). Since neither the absorptivity nor the absorption maxima decrease when MBD is bound to trypsinogen or ribosomes, the conformation of MBD bound to protein is the same as that in water. Other explanations can be invoked to explain this observation, but they are all speculative. Further physical data will be needed to make the correct distinction.

A Hydrophobic Pocket in Ribosomes. The 50S subunit of *E. coli* ribosomes appears to contain a unique hydrophobic binding site for MBD. MBD does not bind to 30S ribosomal subunits alone but does bind very strongly to 70S and to 50S subunits. The dissociation constant is 5 to 9 μM^{-1} and shows little dependence on pH between 6.5 and 8.0 (Table IV). The presence of 30S subunits, *i.e.*, formation of 70S ribosomes from 50S and 30S subunits, has only a minor effect on K_t for MBD binding to 50S subunits. The fluorescence intensity, ΔI, of 50S–MBD complexes is unchanged by addition of 30S subunits at a magnesium concentration favoring association of 30S and 50S subunits (Tissières *et al.*, 1959). Thus formation of 70S particles from 30S and 50S subunits neither creates MBD binding sites in the 30S subunit nor new MBD binding sites in 70S particles between associated 30S and 50S components.

A 70S ribosomal particle from *E. coli* contains $\sim 10^6$ daltons of protein (Tissières *et al.*, 1959) and is distributed into no less than 20 distinct polypeptide chains in the 30S subunit and 30 distinct chains in the 50S subunit (Waller and Harris, 1961; Waller, 1964; Kaltschmidt *et al.*, 1967, 1970; Möller and Castleman, 1967; Möller and Chrambach, 1967; Traub *et al.*, 1967; Traut *et al.*, 1967; Hardy *et al.*, 1969). As judged from titration with MBD of a complete mixture of proteins from the 50S subunit, none of the dissociated, individual proteins is capable of tightly binding MBD. The complete 50S structure is therefore required for binding. Either some protein takes on a particular conformation in the completely assembled subunit such that MBD is tightly bound or the fluorophore is bound in a hydrophobic crevice which is uniquely created by the binding of two or more adjacent proteins onto the 23S RNA. The strength of binding (~ 5 μM^{-1}) further attests to the uniqueness of this hydrophobic binding site. A fluorescent probe tightly bound in this hydrophobic site should prove of considerable value for studying the structure of ribosomes.

Conformational Difference between Trypsinogen and Trypsin. Activation of bovine trypsinogen is initiated by the tryptic hydrolysis of the Lys^6–Ile^7 bond, resulting in release of the amino-terminal negatively charged hexapeptide, Val-(Asp_4)-Lys^6, and active enzyme (Davie and Neurath, 1955; Desnuelle and Fabre, 1955). Neurath *et al.* (1956) first demonstrated that associated with these events there is a conformational change which presumably leads to functional interactions between the active-site seryl and other amino acid residues. Kenner and Neurath (1971), using the fluorescence properties of a dansylaminotyrosyl derivative of trypsinogen, were able to demonstrate the occurrence of a conformational change occurring during transformation of zymogen to enzyme. However, their results were difficult to interpret accurately since the introduction of the dansyl moiety grossly affected the usual kinetics of autoactivation.

The problem is one of demonstrating a conformational change without interfering with the mechanism leading to the change. A noncovalent probe, having some parameter sensitive to conformation and bound in an area of potential change on the zymogen, could be used as a measure of conformation of the zymogen and enzyme separately and thus avoid disruption of the events leading to the change. MBD conveniently shows stronger preferential binding to trypsinogen than to trypsin (Table IV). Furthermore, a decrease in binding follows precisely the kinetics of autoactivation (Figure 7). Concomitant with activation, the conformation is changed such that the hydrophobic site on trypsinogen is either destroyed or distorted in a way that the resulting enzyme only weakly binds the hydrophobic fluorophore. Location of the hydrophobic site has not been possible, but it is noteworthy that the fluorescence measurements made on dansylaminotyrosyl-trypsinogen also indicate that the dansyl group is excluded from a hydrophobic site during the conversion from zymogen to enzyme (Kenner and Neurath, 1971).

The binding of MBD to trypsinogen and trypsin cannot be at the potential or active site of the zymogen and enzyme, respectively. If it were, activation would be competitively inhibited particularly at the initial stages. Also trypsin would be expected to have a binding constant for MBD which is of the same order of magnitude as that for trypsinogen. Both of these are not the case.

Clearly, there is a conformational difference between the hydrophobic sites on trypsinogen and trypsin. The movements of the polypeptide backbone and amino acid side chains may not be large but rather minor reorientations which lead to greater thermodynamic stability of the enzyme. These changes, however small, result in the proper interactions between active-site amino acid side chains and in the distortion or loss of the MBD hydrophobic binding site. Although this site may not be directly involved in enzymatic function, its changes during the transformation of zymogen to enzyme reflect the dynamics of the activation process.

In light of the assumed homology in both primary sequence (Walsh and Neurath, 1964) and three-dimensional structure (Hartley *et al.*, 1965; Sigler *et al.*, 1968; Steitz *et al.*, 1969; Keil *et al.*, 1968) between trypsinogen and chymotrypsinogen, it is useful to compare measurements of conformational

changes during activation of chymotrypsinogen with the measurements that have been made in this work on the trypsinogen system. Using TNS as a hydrophobic fluorescent probe, McClure and Edelman (1967a,b) demonstrated the conformational difference between chymotrypsinogen A and α-chymotrypsin. The changes in fluorescence of the hydrophobic probe were proportional to the appearance of activity. However, in the case of chymotrypsinogen, the fluorescence intensity of TNS increased as α-chymotrypsin appeared, but, in the case of trypsinogen, the fluorescence intensity decreased as enzyme appears. Thus, assuming conformational homology between the two zymogens, it appears that MBD binds to a different hydrophobic site in the trypsinogen molecule than the TNS site in the homologous chymotrypsinogen A.

Under the conditions that trypsinogen binds MBD, neither chymotrypsinogen nor α-chymotrypsin binds the fluorophore. When such analytical tools as MBD show different behavior toward homologous proteins, *i.e.*, bovine trypsinogen and chymotrypsinogen, it is necessary to sound a word of caution about the practice of deducing tertiary structure from primary structure when the tertiary structure of an homologous protein is available. Obviously there are still areas of uniqueness for each protein which make it different from another to which it might be related by divergent evolution.

Conclusion

The present results demonstrate that derivatives of NBD-Cl such as MBD and BBD are of great potential as hydrophobic probes for the study of the dynamics of macromolecular configuration in solution. These compounds possess a high specificity for binding, as shown by the magnitude of the dissociation constants, and a great sensitivity to changes in the polarity of the microenvironment in macromolecules. In addition, the ease with which compounds of the variety of MBD, having a wide range of functional groups, can be synthesized provides powerful new tools for protein and nucleoprotein conformation studies.

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