Fluorescence Properties of Hog Kidney Aminoacylase I

Thomas vom Bruch and Klaus-Heinrich Röhm

Institut für Physiologische Chemie der Philipps-Universität, Karl-von-Frisch-Straße, D-3550 Marburg (Lahn), Bundesrepublik Deutschland

The state of the tryptophan residues of porcine kidney aminoacylase I (EC 3.5.1.14) was investigated by fluorescence spectroscopy and chemical modification. The pH-dependence of the fluorescence emission spectrum of the enzyme indicates that its native conformation prevails between pH 6 and 9.5. Within this range, the ionization of a residue with an apparent pK_a of 7.1 quenches the enzyme fluorescence by about 15%. A similar reduction of fluorescence intensity accompanies the inactivation of aminoacylase I by treatment with N-bromosuccinimide in low excess. This suggests that in both cases a single tryptophyl residue out of eight residues per subunit is affected. Quenching by iodide revealed that, in the native conformation of the enzyme, 5−6 tryptophans per subunit are accessible, while 2−3 are buried within the protein. 8-Anilinonaphthalene-1-sulfonate (ANS) is tightly bound to aminoacylase I (1 mol/mol dimer, $K_d < 1$ μM). ANS binding does not interfere with substrate turnover; the spectroscopic properties of the aminoacylase-ANS complex are consistent with bound ANS being excited by radiationless energy transfer (RET) from buried tryptophyl residues of the enzyme.

Introduction

Aminoacylases are abundant in the kidney and liver of various mammals and were also detected in microorganisms [1]. An aminoacylase from porcine kidney (aminoacylase I, EC 3.5.1.14) is widely used as a reagent to resolve amino acid racemates. Based on the pioneering work of Greenstein and his associates [2], several groups went on to study the molecular and kinetic properties of hog kidney aminoacylase. Bruns and Schulze [3], who were the first to obtain highly purified enzyme, established many of its physical properties. Later, Schneider and his coworkers showed that aminoacylase is a Zn^{2+} metalloenzyme [4] and identified several residues directly or indirectly involved in catalysis [5−8]. Kinetic studies [9−11] provided evidence that catalysis by aminoacylase I follows a linear sequential mechanism of reaction without a covalent intermediate. Here we report the results of a fluorescence spectral study of native and chemically modified aminoacylase I.

Materials and Methods

Enzyme

Aminoacylase I was purified from hog kidney cortex as described elsewhere [11].

Apoenzyme

Native enzyme was treated with 0.5 mM *o*-phenanthroline at pH 6 to remove Zn^{2+}. The resulting solution was dialyzed against a) 20 mM MES/NaOH, pH 6.0, and b) the same buffer containing 1 mM EDTA. In the absence of EDTA the enzyme reabsorbs Zn^{2+}, thereby regaining full activity, whereas in the presence of the chelator the metal-free form is maintained (relative activity < 0.5%).

Enzyme assays

Routinely, aminoacylase activities were measured with 10 mM chloroacetyl-L-alanine in 50 mM MOPS/NaOH buffer, pH 7, at 25 °C. The decrease of absorbance at 242 nm was followed. Practical extinction coefficients were determined from total absorption change. In addition, activities towards acetyl-L-Met were measured by flow injection analysis as described elsewhere [11]. Substrates and inhibitors were kindly provided by Prof. F. Schneider, Marburg.

Determination of tryptophan

The tryptophan contents of aminoacylase I was determined by NBS titration in 8 M urea, pH 4, according to Spande and Witkop [12]. In addition, Trp was assayed by amino acid analysis as described by Ng *et al.* [13]: The amino acids obtained by hydrolysis in the presence of 2-mercaptoethanol were reacted with phenylisothiocyanate and the resulting PTC-derivatives were analyzed by reversed-phase HPLC.

Fluorescence measurements

Excitation and emission spectra were recorded at 25 °C with the Jasco FP-770 spectrofluorimeter (Japan Spectroscopic Co.). Usually both monochromators were set to 3 nm bandwidths. Data processing (smoothing, calculation of difference spectra, correction for dilution effects etc.) was performed numerically using the built-in Jasco software options. Quantum yields Q were estimated according to Kirby and Steiner [12] with free tryptophan ($Q = 0.14$) as the reference.

Quenching experiments

Quenching by iodide ion was studied by adding small volumes of 4 M KI in water (containing 5 mM $Na_2S_2O_3$) to enzyme solutions. KCl at the same concentration was used with the controls. The measured fluorescence intensities were corrected for the volume change caused by titration.

NBS modification

N-Bromosuccinimide was recrystallized from water before use. Aliquots of a freshly prepared 1 mM solution of NBS in water were added to 5−15 μM enzyme solutions (usually in 20 mM MES/NaOH, pH 5.8 at 25 °C), and fluorescence spectra were recorded 1−2 min after addition.

ANS binding

2 μl-Volumes of ANS (1/2 Mg-salt·H_2O, Serva, 0.1 mM in methanol) were successively added with magnetic stirring to 1−2 ml of enzyme solution placed in the fluorescence cell. A 50 μl precision micrometer syringe was used. ANS fluorescence was excited at 285 nm or 375 nm. Control samples (without enzyme) contained the same volume of ANS/methanol in buffer.

Results

Trypthophan contents

A published amino acid analysis of aminoacylase I [5] gave a tryptophan content (6 residues/subunit) that does not agree very well with the reported molar absorption coefficient of the enzyme. We, therefore, reexamined the number of tryptophan residues by NBS-titration and by a recently published amino acid analysis technique that largely avoids oxidative destruction of tryptophan during acid hydrolysis. Both methods, in reasonable agreement, gave values of about 8 tryptophan residues per subunit. The same series of analyses yielded 11−12 Tyr/subunit, rather than 10 as reported in Ref. [5] (data not shown). A comparison of ultraviolet absorption spectra of aminoacylase solutions with amino acid analyses of samples taken from the same batch yielded a specific absorption $A^{1\%}_{280} = 14.3$ which, in turn, corresponds to a molar extinction coefficient $\epsilon = 1.34 \cdot 10^5$ $l \cdot mol^{-1} \cdot cm^{-1}$ (based on a molecular mass of 94,000 for the dimeric native enzyme).

Fluorescence spectra

Representative fluorescence excitation and emission spectra of aminoacylase I are shown in Fig. 1. Both spectra are typical of tryptophan as the main fluorophor. At physiological pH, the excitation maximum was at 284−285 nm, while peak emission intensity was observed at 336 nm. In 50 mM MOPS/NaOH buffer, pH 7.0, the quantum yield Q of the enzyme fluorescence was about 0.67 as compared to a value of $Q = 0.14$ for free Trp.

pH Dependence

The pH-dependence of the aminoacylase fluorescence is illustrated by Fig. 2. In the pH range 4 to 10, the emission intensity varied by less than 10% of the mean. Two different pH effects are discernible: Below pH 6 the fluorescence intensity decreased with pH; at the same time the emission maximum was shifted to higher wavelengths. In contrast, fluorescence intensities fell in a sigmoidal fashion between pH 6 and 9, while the emission maximum was unaffected. The latter data could be fitted by an ordinary titration curve with an apparent pK_a of 7.1. At more alkaline pH, a decline of fluorescence intensity was accompanied by a slight but distinct redshift of fluorescence emission. The low quantum yields
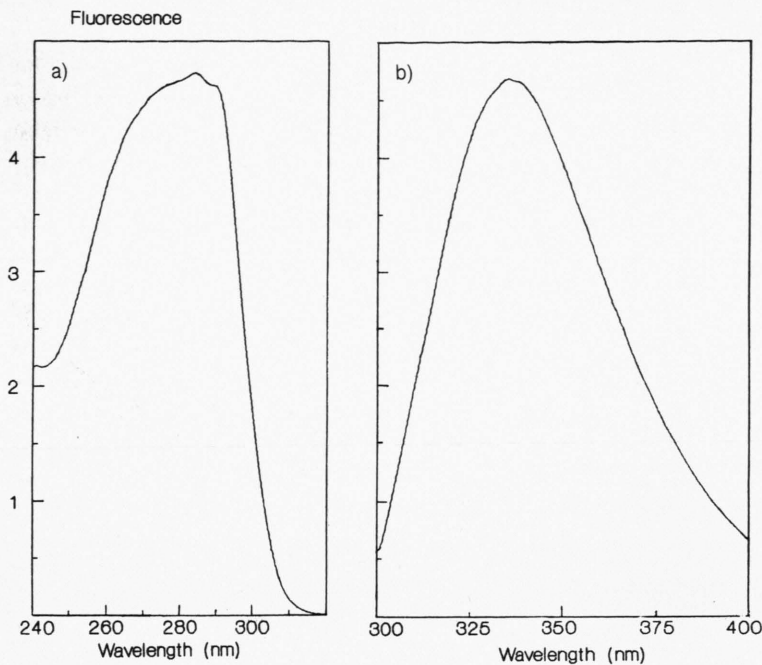
Fig. 1. Excitation and emission spectra of aminoacylase in 50 mM MES/NaOH, pH 6.0 a) excitation spectrum, emission measured at 336 nm b) emission spectrum, fluorescence excited at 285 nm. Fluorescence intensity is given in arbitrary units.
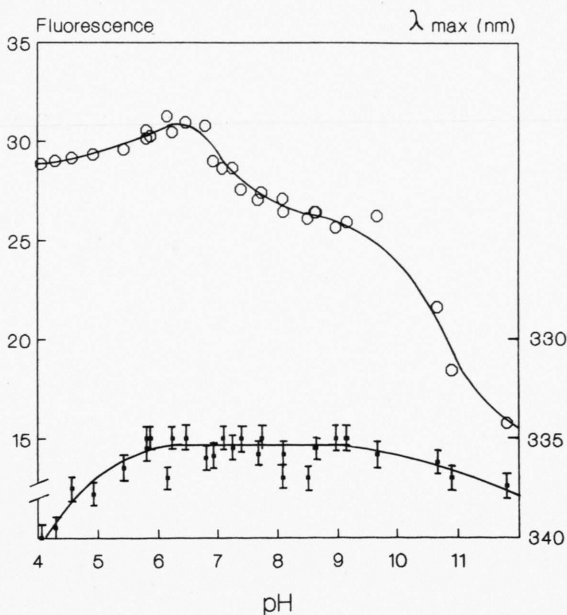

Fig. 2. pH-Dependence of aminoacylase fluorescence. The enzyme sample was dialyzed against a mixed buffer containing MES, MOPS, and TAPS (10 mM each) at pH 5.8. 2.5 ml samples were titrated at 25 °C by successively adding 5 μl volumes of 1 N NaOH, or 1 N acetic acid. After each addition pH was measured with a microelectrode, and emission spectra were recorded. The wavelengths at the emission maxima (■, with bars indicating the estimated standard errors of the readings) and the fluorescence intensities at these wavelengths (○) are plotted vs. pH.

above pH 10 are seen with most proteins; they are mainly due to quenching by ionized tyrosyl residues.

Accessibility of tryptophan residues

The exposition to solvent of protein tryptophan residues can be probed by several methods: First, the emission maximum of the tryptophan fluorescence responds to the polarity of the environment. Second, a variety of low-molecular-weight compounds are capable of quenching the emission of those fluorophors that are accessible to solvent, while the fluorescence of buried residues is hardly affected. Among these chemical quenchers, iodide ion is most frequently employed [15]. Third, in most instances covalent chemical modification of tryptophan also discriminates between exposed and inaccessible residues.

Quenching by KI

Iodide at 0.25 M quenched about 50% of the aminoacylase fluorescence. The concentration dependence of iodide quenching is illustrated by Fig. 3. A modified Stern-Vollmer plot [15] is shown where the reciprocal relative quenching $F_o/\Delta F$ is plotted vs. the reciprocal iodide concentration. The ordinate intercept of such a plot $1/f_a$ is a measure of the "frac-
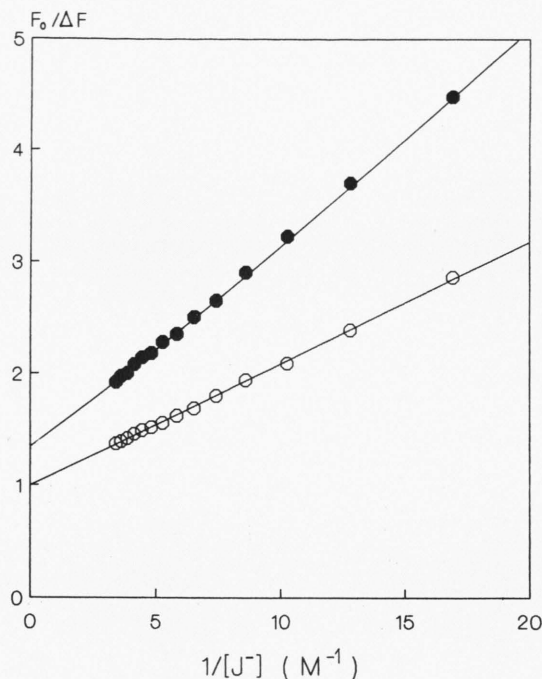
Fig. 3. Quenching of aminoacylase fluorescence by iodide. Increasing amounts of 4 M KI were added at 25 °C to a) free Trp (O), and b) enzyme (●) in 20 mM MES/NaOH, pH 6.0 (see "methods"). Reciprocal relative quenching $F_o/\Delta F$ is plotted vs. reciprocal iodide concentration.

tional accessibility" f_a. By definition, f_a of free tryptophan is 1 (lower curve); with the enzyme tryptophan fluorescence we find $f_a = 1.38$ (upper curve).

Ligands

As already mentioned, the essential Zn^{2+} ions of aminoacylase I can be removed by chelating agents. Difference spectra of apoenzyme and native aminoacylase I (Fig. 4) revealed a distinctly higher fluorescence intensity of apoenzyme in the range of 360−380 nm, while little change was seen below 350 nm. At the same time, fluorescence quenching by 150 mM KI was slightly more effective with apoenzyme (27%) than with native aminoacylase (22%, not shown). On the other hand, p-toluenesulfonyl-L-Phe, a competitive aminoacylase inhibitor ($K_i = 0.15$ mM) at a concentration of 1 mM, had very little effect the fluorescence intensity of the enzyme. A slight decrease of fluorescence (less than 1%) was seen in the range of 340−390 nm while no effect was observed below 340 nm and above 390 nm.

NBS modification

N-bromosuccinimide in relatively low excess has been shown to inactivate aminoacylase by reaction with 1−2 tryptophan residues per subunit [6]. Higher NBS concentrations successively modified the remaining tryptophans and also some of the tyrosine and histidine residues. Unfortunately, these experiments were performed at pH 5.0 and in the presence of 1 M urea, i.e. under conditions now known to denature the enzyme [11]. We, therefore, reexamined the effect of NBS at higher pH and in the absence of urea; in addition, we monitored the fluorescence spectral changes accompanying NBS oxidation (the oxindole derivative of tryptophan formed by NBS treatment is non-fluorescent). In good agreement with Ref. [6] we found that a 20-fold excess of NBS was sufficient to abolish aminoacylase activity (Fig. 5). Only 10−15% of total fluorescence intensity was lost during inactivation. With increasing degree of modification, a gradual blueshift of the emission maximum took place. At NBS/protein ratios of 40 and above the solutions became turbid, indicating the onset of irreversible denaturation.
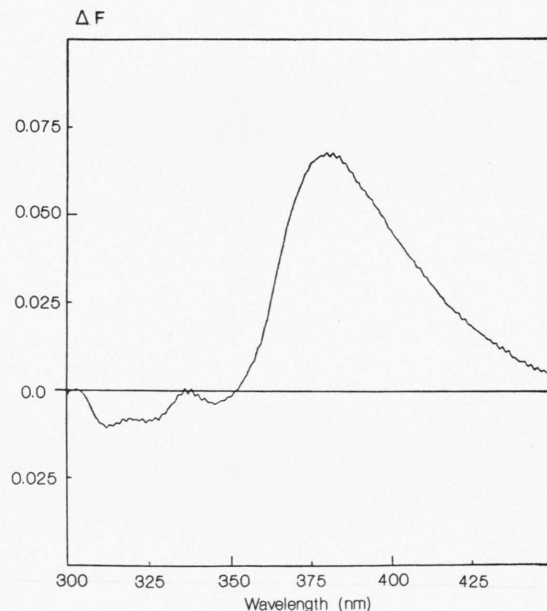

Fig. 4. Difference of the emission spectra of native aminoacylase I and Zn^{2+}-free apoenzyme (F_{apo}-F_{native}). The spectra were normalized to the same enzyme concentration and subtracted numerically. The absolute fluorescence of either preparation at 336 nm was 1.8 fluorescence units (see "methods").
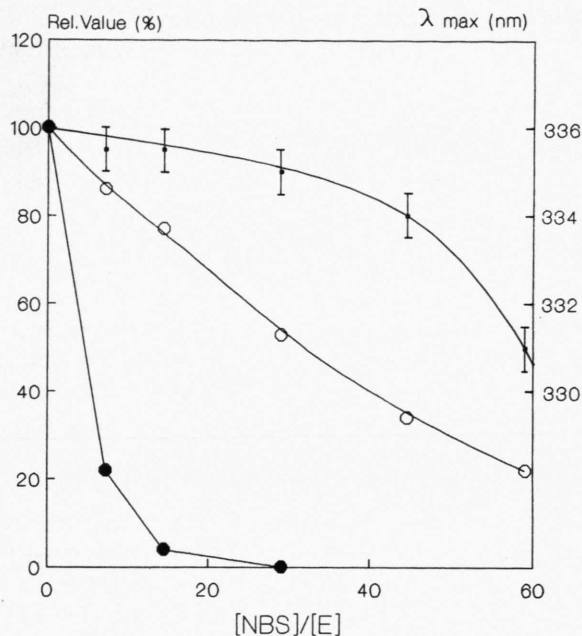
Fig. 5. Modification of aminoacylase I with N-bromosuccinimide. For experimental details see "methods". Relative enzyme activities (●, as percent of untreated controls) and emission intensities (○, also relative to controls) are plotted vs. the excess of NBS over enzyme. In addition, the wavelengths at the emission maxima (■, right ordinate) are given.

ANS binding

8-Anilino-1-naphthalenesulfonate (ANS) is well known for its ability to bind to hydrophobic domains of proteins. While ANS is only weakly fluorescent in water, its quantum yields increase by 1−2 orders of magnitude upon transfer to a solvent of low polarity [16]. We found that ANS binds to aminoacylase at micromolar ligand concentrations. Fig. 6 shows the fluorescence changes accompanying titration of the enzyme with ANS. With increasing amounts of ligand added, the protein fluorescence at 336 nm decreases while a new peak with maximal emission around 465 nm appears. The latter emission is characteristic of ANS in a distinctly hydrophobic environment. Interestingly, the fluorescence of enzyme-bound ANS excited at 285 nm, i.e. at the absorption maximum of the enzyme, was more intense than that excited at the maximum of free ANS at 375 nm. In contrast, the fluorescence intensity of ANS in methanol when excited at 375 nm was 4-fold higher than that measured with excitation at 285 nm.

Saturating concentrations of ANS reduced the protein fluorescence of aminoacylase I by about 25%.

A quantitative evaluation of ANS binding by aminoacylase I is illustrated by Fig. 7. The data are plotted according to Stinson and Holbrook [17]. In this type of graph, the relative concentration of binding sites $n \cdot [E]_t$ is given by the abscissa intercept while the apparent dissociation K_d is obtained from the slope. Fig. 7 suggests that only one ANS molecule is bound to each molecule of dimeric aminoacylase with an apparent dissociation constant of 0.9 μM. Similar results were obtained at other pH values. ANS binding did not interfere with substrate turnover, since no detectable inhibition was observed at ANS concentrations as high as 50 μM. Moreover, 2 mM N-hydroxy-2-aminoheptanoic acid, a strong competitive inhibitor with $K_i = 0.04$ mM, did not prevent ANS binding. It merely led to an about threefold increase of K_d for ANS.

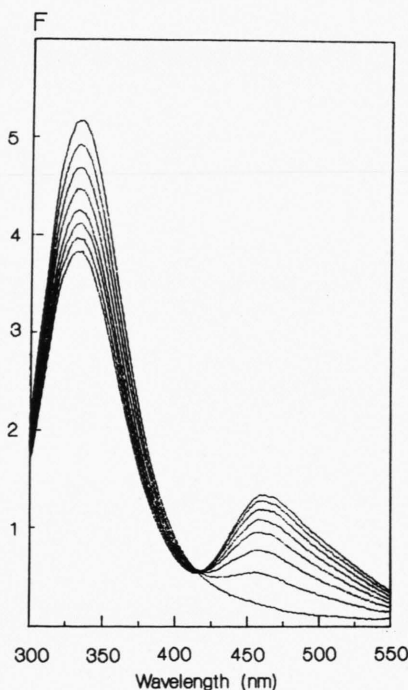Fig. 6. Fluorescence spectral changes during ANS titration. An enzyme preparation previously modified by a 30-fold excess of NBS was used. To 1 ml of enzyme solution (15 μM in 15 mM MES/NaOH, pH 5.8) 20 μl-volumes of 0.1 mM ANS in methanol were successively added. Emission spectra were recorded after each addition (Excitation at 285 nm). The spectra are corrected for the volume change caused by titration.
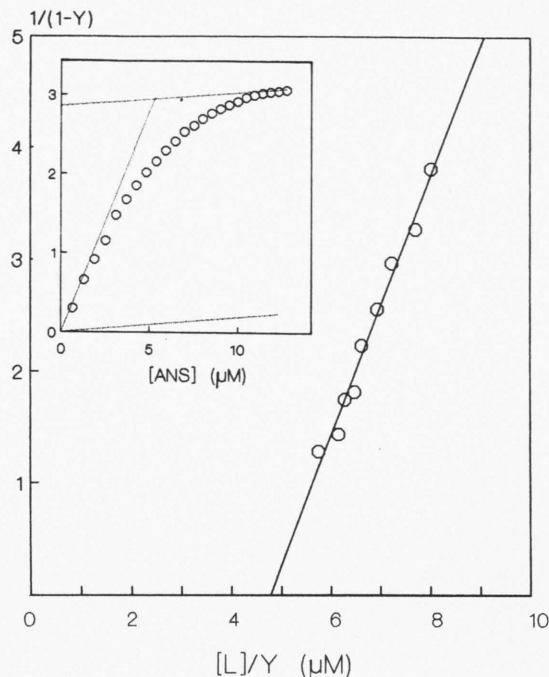
Fig. 7. Quantitative evaluation of an ANS-titration curve. The experiment was performed with 5.8 μM aminoacylase in 50 mM BICIN/NaOH, pH 8.5. Inset: Fluorescence intensities are plotted *vs.* the total concentration of ANS added. The dashed line at the bottom represents the fluorescence of the same concentration of free ANS in buffer without enzyme. The main plate contains the same data plotted according to Stinson and Holbrook (see text).

Partial NBS oxidation also did not impair ANS binding to aminoacylase I. In fact, the spectra shown in Fig. 6 were obtained using an enzyme preparation previously inactivated by a 30-fold excess of NBS. No significant change of the stoichiometry of ANS binding, nor of its binding constant were observed under these conditions.

Discussion

The revised amino acid analysis reported above (16 mol Trp and 22 mol Tyr/mol enzyme) is in much better agreement with the ultraviolet absorption than the earlier values [5]. Still, the molar absorption coefficient calculated from the amino acid composition $(1.19 \cdot 10^5 \, \text{l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1})$ is somewhat lower than the experimental value $(1.34 \cdot 10^5 \, \text{l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1})$. One reason may be that the absorption coeffi-

cients of free and enzyme-bound tryptophan are not the same. Alternatively, the molecular mass of the enzyme used for the calculations may be too low.

The spectroscopic data presented in this paper shed some light on the environment of the tryptophans of aminoacylase I. As already mentioned, the emission maxima of aromatic fluorophors usually reflect the polarity in the vicinity of the emitting residue. So tryptophan in water exhibits maximal emission around 355 nm; with decreasing polarity of the medium the maximum is gradually shifted to shorter wavelengths. In proteins most tryptophan residues are "buried" in the hydrophobic core, hence maximal fluorescence emission is usually observed between 330–340 nm or lower.

Kinetic evidence [11] showed that aminoacylase I is irreversibly denatured at pH's below 5.5. A similar conclusion can be drawn from the effect of acidic pH on the emission spectrum of the enzyme (Fig. 2). A gradual redshift of the emission maximum with decreasing pH indicates that a conformational change takes place that increases the exposure to solvent of tryptophan residues. At alkaline pH, a similar shift does not take place below pH 10, again in agreement with the observation that aminoacylase is stable at least up to pH 9.5. The blueshift of the emission maximum accompanying NBS oxidation of aminoacylase I (Fig. 5) is due to a similar effect: One would expect that the residues most resistant to modification are those most deeply hidden within the protein *i.e.* those emitting at the lowest wavelengths. Indeed, the two tryptophans still unmodified at a 60-fold excess of NBS emit at about 331 nm.

Quenching of the tryptophan fluorescence of enzymes may be exerted by nearby amino acid residues of the protein itself, by bound ligands, or by added solutes. A likely example of the first type of quenching is given by the pH-dependence of aminoacylase fluorescence in the pH range 6–9 (Fig. 2). It appears reasonable to assume that the observed profile is brought about by the dissociation of a group adjacent to one of the tryptophan residues. This process either creates a more effective quencher or triggers a local conformational change to the same effect. The pK_a of 7.1 is consistent with a histidine residue. The presence in the aminoacylase active site of such a residue with a pK_a close to 7 has been demonstrated by Kördel and Schneider [7]. Another ionization event that could be responsible for the observed pH-profile is the dissociation of a Zn^{2+}-bound water molecule in

the active site of the enzyme. The presence of such a residue is suggested by kinetic evidence [11].

Collisional quenching of protein fluorescence by iodide ion can be analyzed by the classical Stern-Volmer law or by extensions of it [15]. However, with proteins containing a multitude of tryptophyl residues only semiquantitative conclusions as to the fraction of accessible residues can be derived. On the condition that each tryptophan contributes approximately the same fraction to the total fluorescence, and assuming a total of 8 residues per aminoacylase monomer, we may estimate that 2–3 of the tryptophyl residues I are largely inaccessible to iodide, while 5–6 can be reached by solutes.

The results of our fluorescence studies of NBS treated enzyme are consistent with data of Kördel and Schneider [6] who quantitized the effect of NBS on aminoacylase I by amino acid analysis. As already mentioned, the oxindole derivative of tryptophan is much less fluorescent than the native amino acid. Fig. 5 shows that, in agreement with the results of iodide quenching, about 60% of the tryptophans reacted with NBS under non-denaturing conditions (i.e. at ratios [NBS]/[E] < 40). A 90% inactivation of aminoacylase I was accompanied by a decrease of enzyme fluorescence by only 10–15%, suggesting but not proving that the loss of activity is due to the oxidation of a single tryptophan residues per subunit. Tyrosine and histidine residues did not react at NBS concentrations sufficient to abolish aminoacylase activity [6].

The binding of ANS to aminoacylase I has several interesting aspects. First, the binding stoichiometry at saturating concentrations of ANS did not exceed 0.9 mol ANS/mol enzyme. Thus we may conclude that only one binding site exists per dimer. Second, the low dissociation constants (< 1 μM) suggest that binding of ANS is highly specific. Nevertheless, the dye does not interact with the active site of aminoacylase I. ANS did not inhibit substrate turnover and competitive inhibitors failed to prevent ANS binding. Third, bound ANS strongly quenched the enzyme fluorescence (by about 25%) while its own fluorescence was most effectively excited at wavelengths that correspond to the excitation maximum of tryptophan. This finding strongly indicates that the fluorescence of bound ANS is excited by radiationless energy transfer (RET) from enzyme tryptophan residues to the bound dye. This type of energy transfer was discovered by Förster [18], its
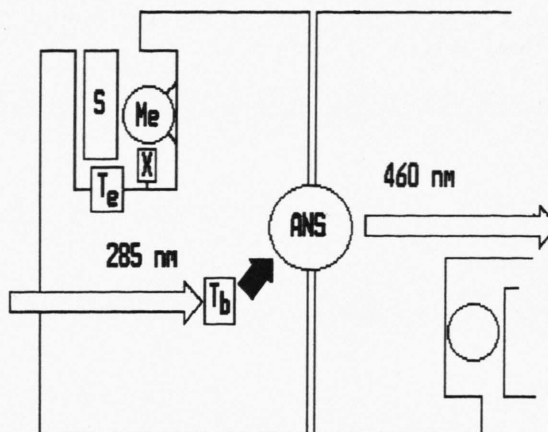

Fig. 8. Possible mode of ANS binding. The scheme illustrates the radiationless energy transfer from a buried enzyme tryptophan (T_b) to bound ANS. The presumed location of an active-site tryptophan (T_e) is also shown. The fluorescence emission if T_e is thought to be affected by the removal of the essential metal ion (Me) as well as by the ionization of a nearby residue X (see text).

efficiency varies with the sixth power of the distance between donor and acceptor. For this reason it is restricted to closely adjacent groups.

Our present data are schematically summarized in Fig. 8. We assume that at least one out of eight tryptophan residues is located in the active site (T_e = exposed tryptophan). It may be the emission of this residue that is affected by the dissociation of an adjacent group X, or by removal of the metal ion Me. We further assume that ANS is bound to a strongly hydrophobic site on the enzyme surface, possibly in the region of subunit contacts. One or more tryptophyl residues in the vicinity of this site are capable of radiationless energy transfer to the ligand, giving rise to an ANS fluorescence higher than that achieved by excitation at its own excitation maximum. None of the exposed tryptophans appears to be involved in binding or radiationless excitation of ANS, as NBS oxidation of the more accessible residues did not abolish ANS binding or energy transfer to the bound fluorophor.

Acknowledgements

[1] I. Gentzen, H. G. Löffler, and F. Schneider, Z. Naturforsch. **35c**, 544−550 (1980).
[2] J. P. Greenstein and M. Winitz, in: Chemistry of the Amino Acids, **Vol. 2,** pp. 1753−1816, Wiley, New York 1961.
[3] F. H. Bruns and C. Schulze, Biochem. Z. **336,** 162−181 (1962).
[4] W. Kördel and F. Schneider, Z. Naturforsch. **32c,** 342−344 (1977).
[5] W. Kördel and F. Schneider, Biochim. Biophys. Acta **445,** 446−457 (1976).
[6] W. Kördel and F. Schneider, Hoppe-Seyler's Z. Physiol. Chem. **357,** 1109−1115 (1976).
[7] W. Kördel and F. Schneider, Z. Naturforsch. **32c,** 337−341 (1977).
[8] H. G. Löffler and F. Schneider, Biol. Chem. Hoppe-Seyler **368,** 481−485 (1987).
[9] I. Y. Galaev and V. K. Svedas, Biochim. Biophys. Acta **701,** 389−394 (1982).
[10] K. H. Röhm and R. L. van Etten, Eur. J. Biochem. **160,** 327−332 (1986).
[11] J. Henseling and K. H. Röhm, Biochim. Biophys. Acta **959,** 370−377 (1988).
[12] T. F. Spande and B. Witkop, Methods Enzymol. **11,** 498−505 (1967).
[13] L. T. Ng, A. Pascaud, and M. Pauscaud, Anal. Biochem. **167,** 47−52 (1987).
[14] E. P. Kirby and R. F. Steiner, J. Biol. Chem. **245,** 6300−6306 (1970).
[15] S. S. Lehrer and P. C. Leavis, Methods. Enzymol. **49,** 222−236 (1976).
[16] L. Stryer, Science **162,** 526−533 (1968).
[17] R. A. Stinson and J. J. Holbrook, Biochem. J. **131,** 719−728 (1973).
[18] T. Förster, Ann. Phys. **2,** 55−75 (1948).