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SHORT COMMUNICATIONS

New method to detect drug-binding protein in muscle using a fluorescence probe

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Kurz *et al.* [1, 2] reported drug binding to muscle and described binding to contractile proteins and lipids, but not to the soluble fraction of muscle homogenate. We reported previously [3] that the anionic drugs FRS* and PBZ bind extensively to the 105,000 *g* supernatant fraction of rat skeletal muscle homogenates. Further, most of the binding was to the second protein fraction (FrII) eluted from the supernatant fraction (Sephadex G-75, Fine, column chromatography). The purpose of the present paper is to report that the anionic fluorescence probe ANS can be used to detect the protein in the supernatant fraction which has affinity for anionic drugs.

ANS, BSA (fraction V, Sigma), RSA (fraction V, Sigma), HXB, PHT, Na-SAA, Na-SDM, Na-SMZ and WAF were obtained commercially. FRS (Hoechst Japan) and PBZ (Nippon Ciba-Geigy) were gifts. They were used without further purification. The 105,000 *g* muscle fraction, which was prepared from 50% male Wistar rat skeletal homogenates as reported previously [3], was fractionated by means of a Sephadex G-75 (Superfine) column and eluted with the same buffer used for homogenization at the rate of 9.5 ml/hr and at 4°. The optical density of 3-ml fractions was determined at 280 nm. The protein concentration was determined according to Lowry *et al.* [4] using BSA as standard. ANS fluorescence was measured with an MPF-4 fluorometer (Hitachi). ANS binding to the fractions was determined fluorometrically, using 380 nm for excitation and 480 nm for emission by titrating 3 ml of the properly diluted fractions by the stepwise addition of 0.3 or 3 mM ANS solution with a microsyringe. The titration data were analyzed by equation 1 according to Wang and Edelman [5].

$$\frac{1}{FI} = \frac{n}{FI_{max}} + \left(\frac{[D]}{K_d} + 1\right)\frac{n}{FI_{max}}\frac{K}{[L]} \qquad (1)$$

where FI, FI_{max} and n are the observed fluorescence intensity, the maximum fluorescence when the binding sites are saturated with ligand, and the number of binding sites respectively. K and K_d are the dissociation constants of ligand (ANS in this case) and a displacer (FRS or PBZ in this case) which binds to the common binding site with ligand, respectively, and $[L]$ and $[D]$ are the ligand and displacer concentrations respectively. A ligand is assumed to emit fluorescence only when it binds to protein. Equilibrium dialysis for protein binding was performed according to the method reported elsewhere [6]. FRS and PBZ were determined as reported previously [3].

The elution pattern of the 105,000 *g* supernatant fraction of 50% muscle homogenates by Sephadex G-75 (Superfine) column chromatography is shown in Fig. 1. The Superfine column fractionated the supernatant better than Sephadex G-75 (Fine) [3] and gave five fractions which were denoted as FrI, FrII, FrIII, FrIV and FrV from the left. Since we reported that the 105,000 *g* supernatant fraction contains

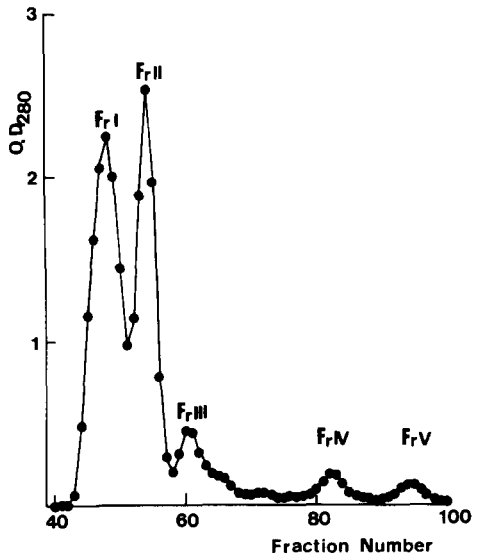

Fig. 1. Elution pattern of 105,000 *g* supernatant fraction of 50% rat skeletal muscle homogenate from Sephadex G-75 (Superfine) (2.65 × 94 cm). The supernatant fraction was eluted with 0.05 M Tris–HCl buffer (pH 7.4) at a flow rate of 9.5 ml/hr and at 4°. Collections were 3 ml/tube and monitored at 280 nm for protein. Fractions were denoted as FrI, FrII, FrIII, FrIV and FrV, from the left.

protein to which anionic drugs bind [3], it was determined whether ANS, an anionic compound, binds to these fractions. ANS emission spectra in these fractions are shown in Fig. 2. The relationships between fluorescence intensities and ANS concentrations, which were obtained by titration, are shown in Fig. 3. FrII showed the strongest intensity. This is fairly consistent with previous observations that the second protein fraction from a Sephadex G-75 (Fine) column showed the strongest binding for FRS and PBZ. It was considered possible that ANS has a common binding site with FRS and PBZ. If so, ANS could be a convenient probe to detect a binding protein for anionic drugs instead of determining drug concentration after equilibrium dialysis.

To examine this possibility, the relationship between fluorescence intensities and ANS concentrations in FrII solutions was determined in the absence and presence of FRS or PBZ. The results are shown in Fig. 4 as double-reciprocal plots according to equation 1. The observation that the data fit straight lines with the same intercept irrespective of the presence of FRS or PBZ provides evidence that they bind to the common binding site with ANS. The values of K and K_d were calculated from the intercepts and slopes in Fig. 4. The dissociation constants were 1.44, 8.8 and 15.8 μM for ANS, FRS and PBZ respectively.

To confirm the fluorescence data, the binding of FRS and PBZ to FrII solution containing 9 mg/ml protein was

* Abbreviations: ANS, 1-anilino-8-naphthalene sulfonate; BSA, bovine serum albumin; FRS, furosemide; HXB, hexobarbital; PBZ, phenylbutazone; PHT, phenytoin; RSA, rat serum albumin; SAA, salicylic acid; SDM, sulfadimethoxine; SMZ, sulfamethoxazole; and WAF, warfarin.

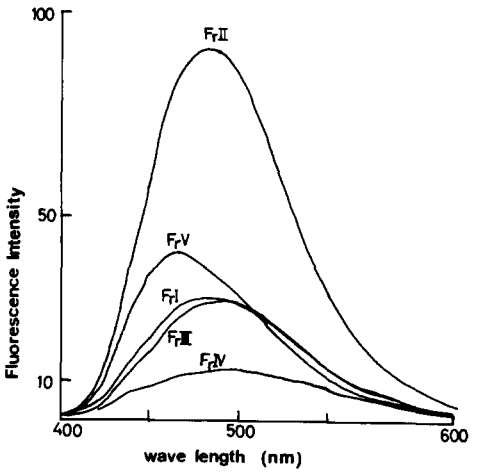

Fig. 2. Emission spectra of ANS in the fractions at 380 nm for excitation. ANS concentration was 10 μM, and protein concentration in each fraction was adjusted to 150 μg/ml. Fluorescence intensities were expressed by relative values.

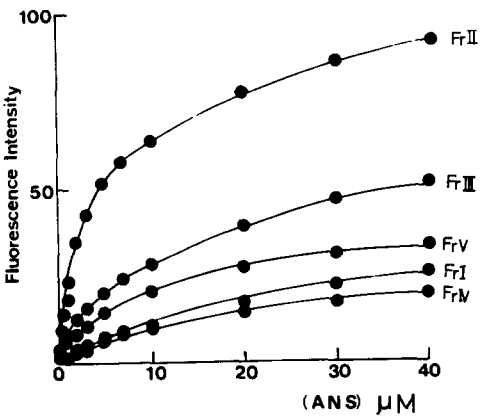

Fig. 3. Fluorescence intensities vs ANS concentrations in each fraction containing 190 μg/ml protein. Fluorescence intensities were measured at 380 nm for excitation and at 480 nm for emission. Fluorescence intensities were expressed by relative values.

determined by equilibrium dialysis at 4°. The dissociation constants were 22.6 and 41.6 μM for FRS and PBZ respectively. Although small differences were found between the two methods, they gave the same rank order for the binding tendency, and the ratios of the K_d values between the two methods were about 2.5 for both FRS and PBZ. Therefore, it is concluded that ANS has a common binding site with anionic drugs such as FRS and PBZ, and that ANS can be used to examine the binding tendency of an anionic drug to protein in FrII. The differences in the results by the two methods were taken to be due to the differences of the methods and the experimental conditions, such as temperature, since fluorescence data determined at room temperature gave lower values of K_d than equilibrium data obtained at 4°. Similarly, Kurz and Fichtl [1] reported that the temperature rise from 4° to 37° increased the binding of chlorpromazine to myosin. Further study on temperature effect on binding is proposed after FrII is more highly purified. This titration method by ANS was then used for other anionic drugs to examine the homogenates. The results are listed in Table 1. The results show that WAF [7], PHT [8] and SAA [9], which are known to bind strongly to albumin, did not bind to FrII and suggest that the protein in FrII is different from albumin.

To examine the properties of the anionic drug binding protein in FrII further, the ANS emission spectrum in FrII was compared with that in RSA solution. The spectra are shown in Fig. 5. Fluorescence intensity in FrII was much smaller than in RSA solution. This supports the above suggestion that anionic drug binding protein in FrII is different from albumin.

Table 1. Binding of anionic drugs to FrII in 105,000 g supernatant fraction of rat muscle homogenate, measured by the ANS titration method

Drug	Binding* (μM)	Drug	Binding
FRS	8.8	PHB	Hardly detected†
PBZ	15.8	HXB	
SDM	3.0	SAA	
		SMZ	
		WAF	

* Binding was expressed by the dissociation constant (K_d).

† Binding was hardly detected up to 20 μM drug.

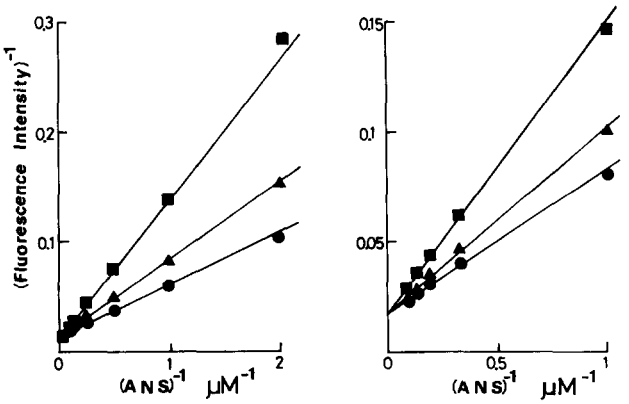

Fig. 4. Double-reciprocal plots of fluorescence intensities and ANS concentrations in FrII solution of 380 μg/ml protein. Fluorescence intensities were measured at 400 nm for excitation, at 490 nm for emission, and at room temperature, and expressed by relative values. Left panel: (●) control; (▲) +5 μM FRS; and (■) +20 μM FRS. Right panel: (●) control; (▲) +5 μM PBZ; and (■) +20 μM PBZ.

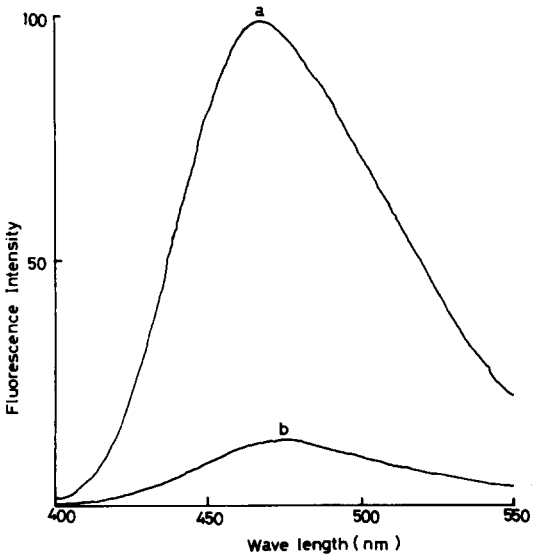

Fig. 5. Emission spectra of ANS in RSA (a) and FrII (b) solutions. ANS concentration was 20 μM and protein concentrations were adjusted to 300 μg/ml in both solutions. Spectra were measured at 380 nm for excitation. Fluorescence intensities were expressed by relative values.

In conclusion, ANS can be used as a convenient probe to detect anionic drug binding protein in muscle, and the ANS titration method is a convenient method to determine the binding constant of anionic drugs. We are now proceeding to purify FrII.

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Effect of 1-β-D-arabinofuranosyl cytosine and hydroxyurea on the repair of X-ray-induced DNA single-strand breaks in human leukemic blasts*

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We have demonstrated previously that ara-C incorporates into DNA of murine and human leukemic cells [1, 2]. The extent of ara-C incorporation into DNA correlates with inhibition of DNA synthesis, and the ara-C residue behaves as a relative chain terminator [3]. These findings are consistent with conformational and hydrogen bonding differences of the arabinose sugar moiety altering reactivity of the 3′-terminus and slowing chain elongation [4, 5]. This disruption of DNA replication by ara-C results in DNA fragmentation [6].

Ara-C inhibits repair of DNA damage of the type induced by u.v. light and certain alkylating agents [7–10]. However, the precise mechanism by which ara-C inhibits repair has not been defined at a molecular level. Ara-C has been shown to inhibit repair of u.v.-induced DNA strand breaks [8]. We recently demonstrated that ara-C incorporates into DNA undergoing repair of u.v. damage [11]. Furthermore, the extent of ara-C (DNA) formation during u.v. repair correlates significantly with cell lethality [11]. These findings thus demonstrated that ara-C incorporates into DNA undergoing large patch repair [12, 13] induced by u.v. irradiation. Since large patch DNA repair synthesis would increase the frequency of ara-C misincorporation, it was of interest to determine whether other agents, such as X-ray, which induce short patch DNA repair would be similarly inhibited by ara-C.

The present study has monitored the effects of ara-C alone and in combination with hydroxyurea on the repair of X-ray-induced single-strand breaks. The results demonstrate that these agents partially inhibit this repair process. However, in contrast to our previous findings with u.v. irradiation, the inhibition of X-ray-induced DNA repair is not associated with detectable incorporation of ara-C. These findings may be relevant when considering therapeutic approaches that employ ara-C in combination with agents that damage DNA.

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Materials and methods

Cells. The human leukemic blasts were maintained in suspension culture as previously described [14]. Chromosomal analysis revealed a human 45 × $^{-}9^{+}$ karyotype.