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Fluorescent Probes for Retinoic Acid Receptors: Molecular Measures for the Ligand Binding Pocket

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Summary: Two fluorescent probes for nuclear retinoic acid receptors (RARs) have been developed, both containing a biologically active retinoid moiety and a fluorescent dansyl moiety, but differing in the length of the spacer arm connecting the two moieties. Both probes bind RARs at their retinoid-binding sites, revealing the usefulness of the compounds as fluorescent RAR probes. By measuring the specific increase of the probes' fluorescence intensity caused by the binding to RARs, the linearized length of the RAR's retinoid-binding pocket could be estimated.

Retinoids (retinoic acid and its biological isosters) have a wide range of effects on morphogenesis and on cell growth and differentiation. The genes for three types of retinoid receptors (RAR-α, β, and γ) have been identified.[1-3] These receptors are members of a large family of ligand-dependent transcription factors sharing highly conserved DNA-binding domains; i.e., the steroid/thyroid receptor superfamily.[4,5] Although the RAR-encoding genes have been characterized, there is limited information concerning the RAR-proteins themselves. The lack of success in characterization/purification of RARs is considered to be due to their instability and low concentration. To overcome these difficulties, superior probes for RARs are mandatory. Analysis of RAR-proteins, especially that of the RAR-ligand binding site, is expected to be possible by the use

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of structure-activity relationships of a large number of retinoid analogs and several designed RAR-probes.

One possible approach to develop superior RAR probes is to produce fluorescent probes. Recently, we have developed fluorescent/photoaffinity RAR labeling probes based on our previous studies of synthetic retinoids.[6-10] In this paper, we describe the development of two fluorescent RAR probes and their usage as molecular measures for estimating the linearized length of the RAR's ligand-binding pocket.

MATERIALS AND METHODS

2-[3-(5-Dimethylaminonaphthalene-1-sulfonyl)aminopropyl-1-oxy]-4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-carboxamido]benzoic Acid (DAM-3)

DAM-3 was parepared as previously described.[10]

Dansyl Undecanoic Amide of 2-(Aminopropyl-1-oxy)-4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]-benzoic Acid (DAM-15)

Dansyl undecanoic acid was converted to its activated ester by N-hydroxysuccinimide treatment. Then the activated ester was condensed with 2-(aminopropyloxy)-4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]benzoic acid[11] to give DAM-15. mp.45-46℃. FAB/MS ($M+H^+$): 841. ^{1}H-NMR ($CDCl_3$): 1.00-1.10 (m, 8H), 1.13-1.23 (m, 6H), 1.28 (s, 6H), 1.30 (s, 6H), 1.56 (m, 2H), 1.70 (s, 4H), 2.06 (m, 2H), 2.23 (t, J=7.5 Hz, 2H), 2.83 (q, J=6.5 Hz, 2H), 2.88 (s, 6H), 3.47 (q, J=5.5 Hz, 2H), 4.26 (t, J=6.0 Hz, 2H), 5.13 (s, 1H), 7.11 (dd, J=2.0, 8.5 Hz, 1H), 7.14 (s, 1H), 7.17 (d, J=7.5 Hz, 1H), 7.35 (d, J=8.5 Hz, 1H), 7.49 (dd, J=8.0, 8.0 Hz, 1H), 7.51 (dd, J=8.0, 8.0 Hz, 1H), 7.64 (dd, J=2.0, 8.0 Hz, 1H), 7.90 (d, J=2.0 Hz, 1H), 7.92 (d, J=2.0 Hz, 1H), 7.97 (d, J=8.5 Hz, 1H), 8.20 (dd, J=1.0, 7.5 Hz, 1H), 8.29 (d, J=8.5 Hz, 1H), 8.52 (d, J=8.5 Hz, 1H), 8.59 (d, 1H).

Preparation of RAR-α Fraction from HeLa Cells

A HeLa whole cell extract was prepared as previously described.[11-16] The major RAR contained in the crude extract thus prepared was RAR-α with less than 20% contamination by RAR-β.[13] The extract was separated by Q-Sepharose Fast Flow (Pharmacia) column chromatography, and the fractions containing RAR-α were used for the experiments.

Binding Competition Assay

A binding competition assay using tritium-labeled retinoid, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)benzoic acid, ([^{3}H]Am80) was performed as previously described.[11-14]

Binding Measurement of Fluorescent Probes

RAR-α fraction (1 mg protein/ml: 20 mM Tris pH 8.0 - 0.15 M NaCl) was mixed with 1 μM of DAM-3/DAM-15 in the presence or absence of 100 μM of various competitors. The fluorescence intensity of the mixture was directly measured (350 nm: excitation, 520 nm: emission).

RESULTS AND DISCUSSION

The structures of the DAMs are shown in Fig. 1. Each DAM consists of a biologically active retinoid moiety derived from 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-

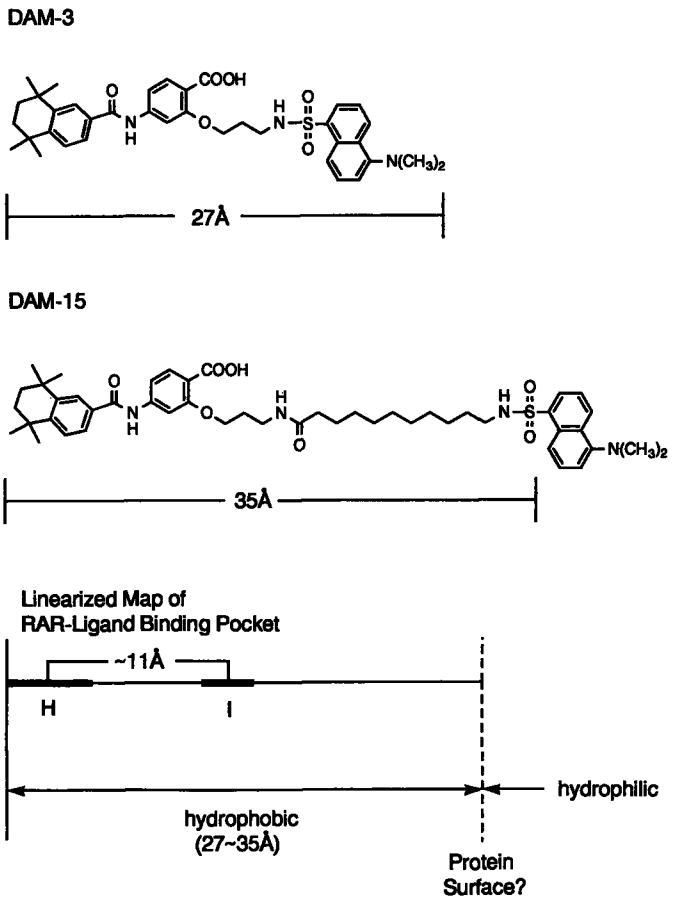

Fig. 1. Structures of DAM-3 and DAM-15, and the Linearized Map of the RAR-α 's Ligand-Binding Pocket.
H and I: The hydrophobic interaction site and the ionic interaction site, respectively.[6, 7, 10, 17]

carboxamido]benzoic acid (Am580)[6] and a fluorescent dansyl moiety; however, the length of the spacer arm which connects the two moieties is different, i.e., the linearized lengths of the spacer arms of DAM-3 and DAM-15 are approximately 8Å and 23Å, respectively.

The [^{3}H]Am80 binding competition assay showed that DAMs bind RAR-α in a mutually competitive manner with respect to Am80 (data not shown). Specific binding of [^{3}H]Am80 was completely inhibited by addition of 1 mM of DAM-3 or DAM-15. Based on the DAM-3/DAM-15 concentrations necessary to cause 50% inhibition of the [^{3}H]Am80 binding, their binding constants were estimated to be similar (of the order of 10^6 M^{-1}). In accordance with this, DAM-3 showed HL-60 cell differentiation induction activity with an ED_{50} value of the order of 10^{-8} M (Kawachi et al., unpublished results).

Table I. Relative Fluorescence Intensity of DAM-3 and DAM-15

Solvent	Relative Fluorescence Intensity*	
	DAM-3	DAM-15
H_2O	1.00	3.12
10% ethanol/H_2O	1.25	3.25
25% ethanol/H_2O	3.38	3.50
50% ethanol/H_2O	7.00	6.50
75% ethanol/H_2O	10.63	9.94
ethanol	15.25	11.13
acetone	16.38	10.63
chloroform	18.38	12.13
20mM Tris (pH 8.0)/0.15 M NaCl	0.75	1.87
20mM Tris (pH 8.0)/0.30 M NaCl	0.50	1.50
20mM Tris (pH 8.0)/0.60 M NaCl	0.38	1.62
20mM Tris (pH 8.0)/1.0 M NaCl	0.50	2.87

* The fluorescence intensity of DAM-3 in H_2O was defined as 1.00.

DAMs showed strong, hydrophobicity-dependent fluorescence (Table I). It might be expected that the fluorescence intensity of DAMs could be increased by binding to RAR-α, if the ligand-binding site is in a hydrophobic environment. This consideration suggests the possibility that RAR-α can be detected by measuring the specific increase of relative fluorescence intensity of DAMs caused by the addition of RAR-α. In fact, as shown in Fig. 2, the fluorescence intensity of DAM-3 was greatly increased by the addition of the RAR-α fraction. This RAR-α-dependent increase of fluorescence intensity was diminished by the addition of Am80, retinoic acid, or other retinoids as a competitor. The results indicate that the RAR-α-dependent increase of DAM-3's fluorescence intensity is due to its binding to RAR-α at the specific ligand-binding site. Though a similar fluorescence intensity increase of DAM-3 was observed upon addition of bovine serum albumin (BSA), this increase was not reduced by the addition of the competitors, indicating that this BSA-dependent fluorescence increase was due to non-specific binding of DAM-3 to the protein.

On the other hand, though DAM-15 also showed an RAR-α-dependent increase of its fluorescence intensity, this increase was not reduced by the addition of competitors (Fig. 2). Therefore, the RAR-α-dependent fluorescence increase of DAM-15 is

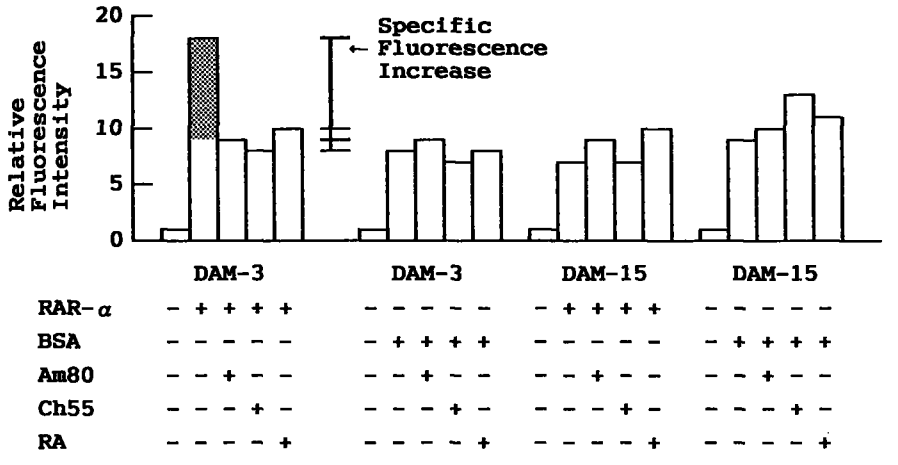

Fig. 2. RAR-α -Dependent Increase of Relative Fluorescence Intensity of DAM-3 and DAM-15.
The relative fluorescence intensity of DAM-3/DAM-15 in the presence or absence of RAR-α fraction/BSA, and the effects of the addition of various competitors are shown. Fluorescence intensity of DAMs in buffer were normalized to be 1.00. RA: retinoic acid. Ch55: (E)-4-[3-(3,5-di-tert-butylphenyl)-3-oxo-1-propenyl]benzoic acid.[9] Shaded: Specific fluorescent increase.

due to its non-specific binding, but not to its specific binding to the RAR-α 's ligand-binding site. Since both DAM-3 and DAM-15 have been shown to bind specifically to the RAR-α 's ligand-binding site, the lack of an RAR-α -dependent specific increase of DAM-15's fluorescence can be interpreted in terms of the position of the dansyl moiety in the RAR-α /DAM-15 complex; i.e., probably, the DAM-15's dansyl moiety is positioned beyond the RAR-α protein's surface when DAM-15 specifically binds to the ligand-binding site. This consideration, as well as our previous studies, which show that the RAR's ligand-binding site consists of a hydrophobic interaction site located inside the protein and an ionic interaction site located near the surface of the protein,[7,10] allowed us to estimate the linearized length of the RAR-α 's ligand-binding site (Fig. 1). Since DAM-3's and DAM-15's dansyl groups are probably situated inside and outside of the RAR-α 's ligand-binding site, respectively, the linearized length of the ligand-binding site can be tentatively (simply using linear conformations of DAMs as molecular measures) estimated to be longer than ~ 27 Å and shorter than ~ 35 Å (Fig. 1). Of course, this estimation is over-simplified (i.e., the RAR-α -bound DAMs' conformation is not likely to be linear and the RAR-α 's ligand-binding site may not be bay-like in shape), and the depth of the ligand-binding pocket is likely to be shorter than the estimated linearized length. In spite of

this uncertainty, the results presented here offer useful interim information concerning the shape/size of the RAR's ligand-binding site, given that X-ray crystallography of RAR/ligand complex has not been possible yet.

In conclusion, we have developed two fluorescent RAR probes, DAM-3 and DAM-15, one of which (DAM-3) can be used for probes to detect RAR-α (and possibly other RAR-subtypes) without separation of free probes from RAR-bound probes. Using these probes, the linearized length of the RAR-α 's ligand binding pocket was estimated.

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