Subunit exchange of lens α-crystallin: a fluorescence energy transfer study with the fluorescent labeled αA-crystallin mutant W9F as a probe

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Abstract A Trp-free αA-crystallin mutant (W9F) was prepared by site-directed mutation. This mutant appears to be identical to the wild-type in terms of conformation (secondary and tertiary structures). W9F was labeled with a sulfhydryl-specific fluorescent probe, 2-(4′-maleimidylanilino) naphthalene-6-sulfonate (MIANS), and used in a subunit exchange between αA- and αA-crystallins as well as between αA- and αB-crystallins, studied by measurement of fluorescence resonance energy transfer. Energy transfer was observed between Trp (donor, with emission maximum at 336 nm) of wild-type αA- or αB-crystallin and MIANS (acceptor, with absorption maximum at 313 nm) of labeled W9F when subunit exchange occurred. Time-dependent decrease of Trp and increase of MIANS fluorescence were recorded. The exchange was faster at 37°C than at 25°C. The energy transfer efficiency was greater between homogeneous subunits (αA-αA) than between heterogeneous subunits (αA-αB). A previous exchange study with isoelectric focusing indicated a complete but slow exchange between αA and αB subunits. The present study showed that the exchange was a fast process, and the different energy transfer efficiencies between αA-αA and αA-αB indicated that αA- and αB-crystallins were not necessarily structurally equivalent.

Key words: α-Crystallin; αA-Crystallin mutant; Trp fluorescence; Fluorescence resonance energy transfer; Intermolecular exchange; Circular dichroism

1. Introduction

The mammalian lens soluble protein consists of three major components: α-, β- and γ-crystallins. α-Crystallin, which makes up over 40% of lens proteins, comprises two highly homologous subunits: αA and αB. The two subunits associate non-covalently to form an aggregate with a molecular mass of approximately 800 kDa [1]. The α-crystallin becomes increasing insoluble with age and cataract formation. Study of the mechanism of insolubilization has been hindered by a lack of knowledge about the three-dimensional structure and the scarcity of pure, unmodified α-crystallin. The recent surge in the use of recombinant techniques in molecular biology has greatly facilitated such studies [2–6]. Especially useful is site-specific mutation in the study of protein folding and conformation as well as protein interaction. Recently, a Trp-free αA-crystallin mutant, W9F, in which the sole Trp residue was replaced with Phe, was prepared [3] and used to examine conformational change of substrate proteins in a chaperone binding study [7].

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The heterogeneous nature of α-crystallin poses some interesting queries. One of these, subunit exchange, appears most interesting. Such subunit exchange has been investigated previously by means of isoelectric focusing electrophoresis (IEF) in the native form [8–10]. These studies indicated a slow subunit exchange between αA- and αB-crystallins. One of the effects due to subunit exchange was stabilization of αB- by αA-crystallin [10]. More recently, a study of fluorescence resonance energy transfer between two fluorescent labeled αA-crystallins indicated rapid exchange among αA-crystallin subunits [11]. In the present report, we extend such subunit exchange to a heterogeneous αA-αB system with the use of an αA-crystallin mutant (W9F) labeled with a sulfhydryl-specific fluorescent probe. Energy transfer between wild-type αA- or αB-crystallin and labeled mutant could be measured without the use of two labeled fluorescent probes.

2. Materials and methods

2.1. Preparation of Trp-free α-crystallin mutant (W9F)

pAED4-αA, a double-stranded supercoiled expression vector containing αA-crystallin cDNA, was prepared previously; the αA gene was isolated from a human lens epithelial cell cDNA library and cloned into the pAED4 expression vector [6]. Site-directed mutagenesis was carried out with use of a QuickChang mutagenesis kit (Stratagene, La Jolla, CA). In brief, a synthetic oligonucleotide primer bearing the desired mutation sequence and its complementary oligonucleotide primer were used to perform a polymerase chain reaction (PCR) that amplified pAED4-αA. The PCR was performed with *Pfu* DNA polymerase, which replicates plasmid strands with high fidelity but without displacing the mutant primer. Upon incorporation of the primer, a mutated plasmid was generated. The PCR products were treated with *Dpn*I to digest parental DNA isolated from *Escherichia coli*. The PCR-synthesized mutation-containing DNA, which is non-methylated and resistant to *Dpn*I, was transformed into *E. coli* (XL-1 Blue supercompetent cell). Twelve colonies were picked up from the transformants and were identified by enzyme digestion; the mutagenesis efficiency was found to be 66%. To confirm the desired mutation, the DNA sequence of the mutant was determined.

Two primers, forward (5′-GACCATCCAGCACCCCTTCTTTA-AACGCACCCTGGGGCCC-3′) and reverse (5′-GGGCCCCAGGG-TGCGTTTAAAGAAGGGGTGCTGGATGGTC-3′), were custom synthesized by Gibco-BRL Life Technologies. In the primer sequence, the Trp-9 codon was replaced by a phenylalanine codon (W9F).

E. coli strain BL21(DE3) was transformed with pAED4-αA(W9F) in the expression of the αA-crystallin mutant. The procedures used for expression and for protein purification have been detailed elsewhere [6]. Protein concentration was determined with a known extinction coefficient based on aromatic amino acid composition [12].

2.2. SDS-PAGE

SDS-PAGE was performed in a slab gel (15% acrylamide) [13] under reducing conditions with a constant voltage of 200 V for 1 h. The gels were stained with Coomassie brilliant blue R-250.

2.3. Circular dichroism (CD) measurements

CD spectra were measured with an Aviv circular dichroism spectrometer (model 60 DS) [14]. The reported CD spectra are the average

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of 5 scans, smoothed by polynomial curve fitting. CD data were expressed as molar ellipticity in deg-cm^2 dmol^{-1}, with 115 as residue molecular weight.

2.4. Fluorescence measurements

Fluorescence was measured with a Shimadzu spectrofluorometer (model RF-5301PC). Trp fluorescence was scanned at an excitation wavelength of 295 nm. The SH-specific fluorescent probe, 2-(4′-maleimidylanilino) naphthalene-6-sulfonate (MIANS), was used for labeling [15]. A 10-fold excess of MIANS (50 μM) was added to α-crystallin solutions (0.1 mg/ml in 0.05 M phosphate buffer, pH 7.6) and incubated at 4°C overnight. The excess MIANS was then dialyzed out. Emission was scanned with an excitation wavelength at 313 nm.

For experiments of fluorescence resonance energy transfer, the labeled αA-crystallin mutant was mixed with unlabeled αA- or αB-crystallin. Time-dependent emission spectra were obtained at an excitation wavelength of 290 nm. The efficiency (E) of energy transfer between donor probe (Trp) and acceptor probe (MIANS) was determined by measurement of the fluorescence intensity of the donor both in the absence (F_d) and in the presence (F_{da}) of acceptor, as shown in:

$$E = 1-(F_{da}/F_d) \quad (1)$$

The efficiency is a function of the inverse sixth power of the distance between donor and acceptor [16]. Since the critical distance for energy transfer is in the range of 20–50 Å and the size of the α-crystallin aggregate is 150–200 Å, the energy transfer efficiency reflects a sensitive detection of either intermolecular exchange or intermolecular interaction.

3. Results

3.1. Characterization of the αA-crystallin mutant

DNA sequencing showed that we succeeded in producing a Trp-free αA-crystallin mutant. The SDS gel had one single band at 20 kDa (Fig. 1) and little Trp fluorescence was evident for the mutant (Fig. 2). The low-level emission that was detected may have come from other aromatic amino acids, such as tyrosine and phenylalanine.

CD spectra of the mutant and wild-type αA-crystallin are shown in Fig. 3. The far-UV CD spectra for the mutant and wild-type were the same, a finding indicating no change in secondary structure. The difference in the near-UV CD might have reflected either the lack of Trp residue in the mutant or a subtle change in tertiary structure, and was further studied by MIANS fluorescence. Upon incubation with MIANS and then dialysis, the degree of labeling was estimated to be approximately 1 molar ratio for both wild-type and mutant αA-crystallins. The MIANS labeled mutant did not differ from the wild-type αA-crystallin in the emission maximum or emission intensity (Fig. 4); SH groups did not become more exposed upon mutation. These spectroscopic measurements, therefore, showed that the W9F mutant did not change conformation. No MIANS fluorescence and so no labeling was observed for αB-crystallin; it did not have any SH groups. The MIANS labeled W9F mutant was used in the energy transfer study.

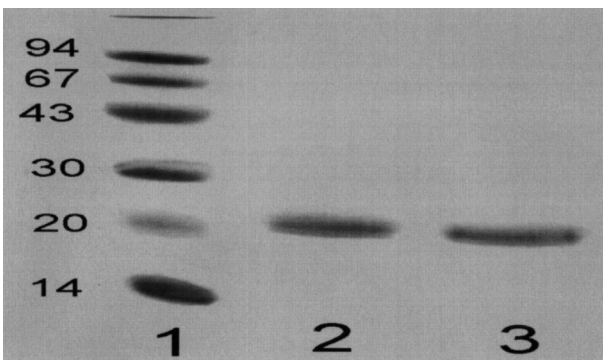

Fig. 1. SDS-PAGE of wild-type αA-crystallin and mutant W9F. Lane 1, standard markers; lane 2, W9F; and lane 3, wild-type αA-crystallin.

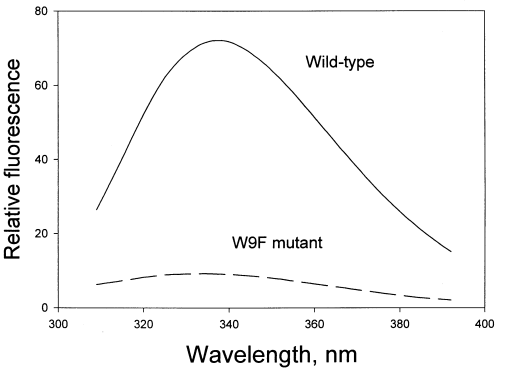

Fig. 2. Trp fluorescence of wild-type αA-crystallin and mutant W9F. Excitation wavelength, 295 nm; protein concentration, 0.1 mg/ml.

3.2. Energy transfer

Depletion of the single Trp amino acid (Trp-9) in αA-crystallin provides a convenient way to study intermolecular exchange among subunits. The MIANS labeled mutant can be used to study the exchange between mutant and wild-type αA-

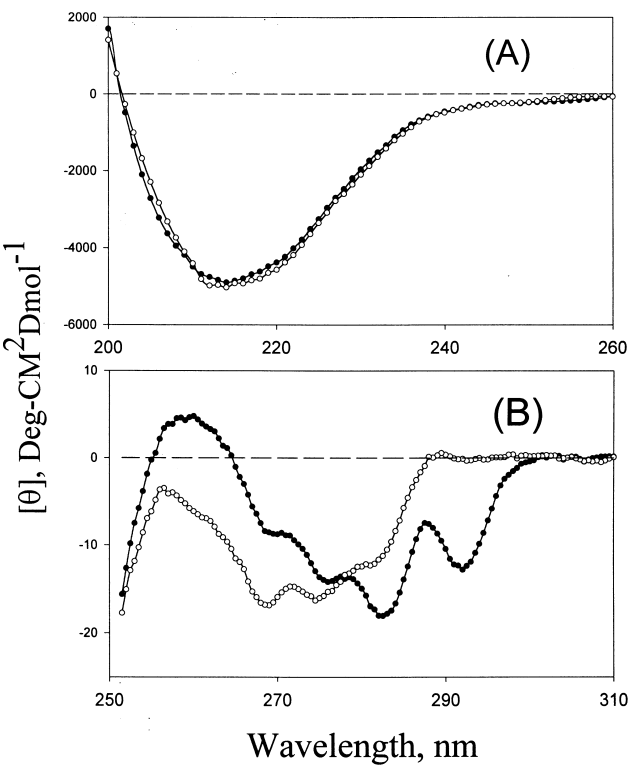

Fig. 3. CD spectra of wild-type αA-crystallin and mutant W9F (A) in the far-UV region and (B) in the near-UV region. Protein concentrations were 0.5 mg/ml (cell path length, 2 mm) and 1.0 mg/ml (cell path length, 10 mm) for the far-UV and near-UV CD, respectively.

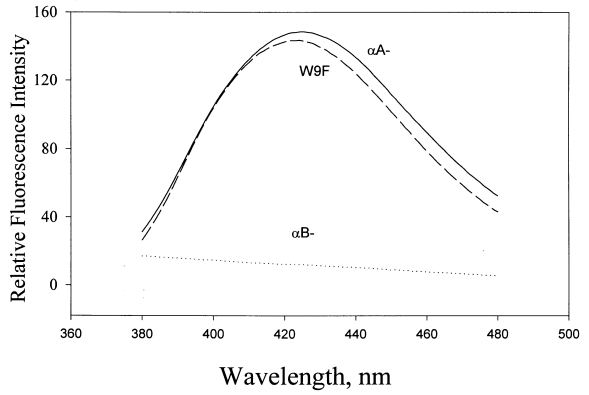

Fig. 4. Fluorescence of MIANS labeled wild-type αA-crystallin and W9F mutant. Excitation wavelength, 320 nm; protein concentration, 0.1 mg/ml.

or αB-crystallin by assessing fluorescence resonance energy transfer. At an excitation wavelength of 290 nm, emission intensity of Trp decreased and emission intensity of MIANS increased if energy transfer occurred.

Fig. 5 shows representative spectra for the decrease in Trp fluorescence and increase in MIANS fluorescence after 8 h of incubation at 25°C for the mixture of labeled mutant and wild-type αA-crystallin. A similar, but less extensive change was observed with αB-crystallin. Some energy was transferred from the donor (Trp) to the acceptor (MIANS). The data for the increase in MIANS intensity with incubation times at 25 and 37°C are plotted in Fig. 6. The exchange was faster at 37°C than at 25°C, and the increase of fluorescence intensity was greater for homogeneous subunits (αA-αA) than for heterogeneous subunits (αA-αB). The curves of intensity increase became flattened in the first 30 min and 2–3 h for αB-crystallin at 25 and 37°C, respectively, but continued to increase until over 2–3 h and 24 h for αA-crystallin at the corresponding temperatures. The curves for decreased Trp intensity were similar (data not shown). The efficiency of energy transfer calculated with Eq. 1 was 0.65 for αA- and 0.13 for αB-crystallin at room temperature after 24 h of incubation.

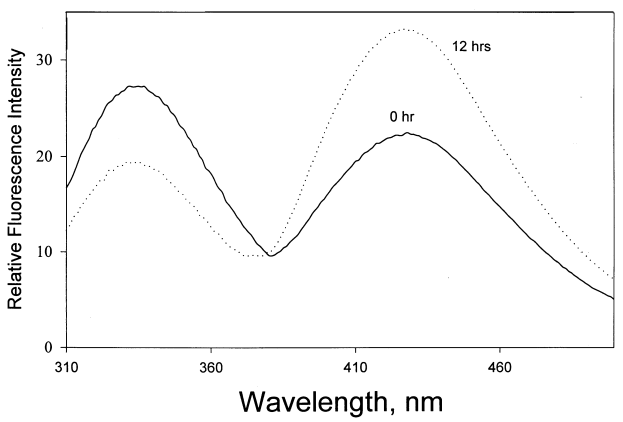

Fig. 5. Representative spectra of decrease in Trp and increase in MIANS fluorescence intensity for the mixture of MIANS labeled W9F and wild type αA-crystallins. Spectra were taken 12 h after mixing at 25°C with excitation wavelength of 290 nm.

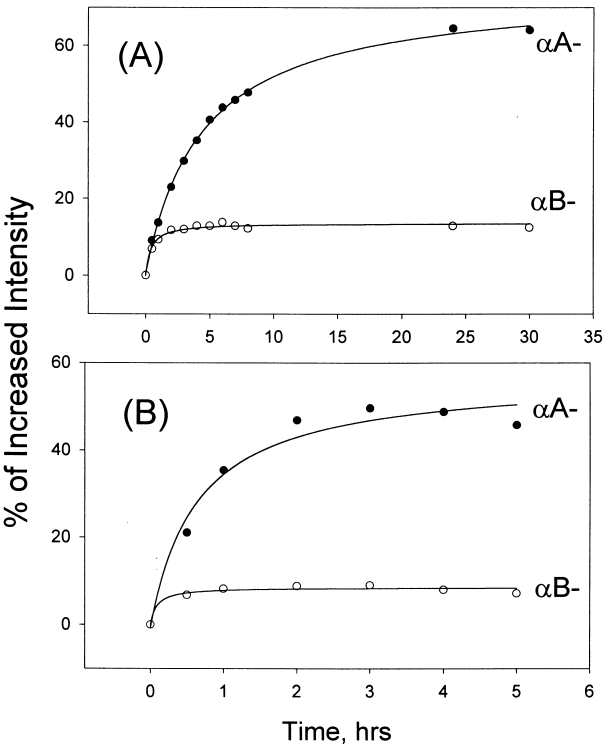

Fig. 6. Time dependent percentage increase in MIANS fluorescence intensity: a mixture of MIANS labeled W9F mutant and wild-type αA-crystallins incubated at 25°C (A) and at 37°C (B).

4. Discussion

It is surprising that the replacement of hydrophobic Trp with Phe did not change the conformation of αA-crystallin; Trp-9 is in the N-terminal domain, which participates in high molecular weight aggregation [2]. Previous reports also indicated little difference in conformation and chaperone-like activity between W9F and wild-type αA-crystallin [3,7]. Apparently, replacing with the less hydrophobic Phe did not change much of the overall hydrophobicity. The W9F mutant provides an ideal model not only for chaperone studies [7] but also in subunit exchange as shown in the present study. An-Other possible study is photooxidation. Since the mutant lacks a Trp residue, the role of Trp oxidation during UV irradiation may be explored and is currently being studied in our lab.

The dynamic structure of α-crystallin was first reported by van den Oetelaar et al., who used IEF to study calf lens αA- and αB-crystallins [8]. Recently, we also used IEF to study subunit exchange but used recombinant αA- and αB-crystallins [9]. Recombinant αA- and αB-crystallins were in the native form [6] while calf lens αA- and αB-crystallins were isolated under urea denaturation. Urea treatment was reported to change conformation of the refolded α-crystallin [17]. Subunit exchange was a time and temperature dependent reaction; the complete exchange between αA- and αB-crystallins required 24 h at 37°C [9,10]. At any ratio, αA- and αB-crystallins were able to become one entity through exchange, as shown by the merging of the two separate bands into one hybrid band [10]. The present energy transfer study indicated

that exchange was a fast process; energy transfer reached saturation within a few hours. The greater energy transfer between homogeneous subunits than between heterogeneous subunits probably reflected greater efficiency rather than greater exchange. This finding is difficult to explain in light of previous reports that the αA and αB subunits are structurally equivalent and occupy equivalent sites in the α-crystallin aggregate [8,9]. The rapid exchange rate was also reported by Bova et al. [11] with energy transfer between two labeled αA-crystallins. The difference in exchange rate observed by IEF and energy transfer may have been due largely to different sensitivity of detection. The rate of energy transfer (in ns) is obviously much faster than the rate of subunit exchange (in h). One possible drawback in energy transfer measurement is that since the size of α-crystallin (150–200 Å) is greater than the critical distance for energy transfer (20–50 Å), the distance between Trp in one αA-crystallin and MIANS in another may not always be within the critical distance due to the slow exchange rate.

Another possible explanation for the greater energy transfer between homogeneous than heterogeneous subunits is that, once subunit exchange occurs, MIANS bound to cysteine in the W9F mutant may undergo exchange with unlabeled SH in wild-type αA-crystallin, which provides a system for energy transfer between Trp and MIANS within the same αA-crystallin subunit. This kind of exchange cannot occur with αB-crystallin because it lacks cysteine. Whether the explanation is true or not can be proved by preparing a cysteine-free αA mutant and performing a similar exchange study.

In conclusion, we have demonstrated that subunit exchange is a fast process measured by energy transfer whose study is facilitated by the αA-crystallin mutant. Energy transfer is more efficient between homogeneous (αA-αA) than heterogeneous (αA-αB) subunits.

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