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Titration of Recombinant Aequorin with Calcium Chloride

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The photoprotein aequorin emits light in the presence of a trace of Ca^{2+}. The primary structure of the protein indicates the presence of three Ca^{2+}-binding sites, whereas the luminometric titration of heterogeneous natural aequorin with Ca^{2+} has shown that the light emission takes place by the binding of two Ca^{2+} ions. In the case of recombinant aequorin, which is more suitable for quantitative studies, the titration with Ca^{2+} monitored by a Ca^{2+}-sensitive electrode revealed that the photoprotein can bind more than two, most likely three, Ca^{2+} ions, and the luminometric titration conclusively showed that the luminescence is triggered by the first two Ca^{2+} ions bound. The affinity of recombinant aequorin for the first two Ca^{2+} ions, which are essential for light emission, was about 20 times stronger than that for the third Ca^{2+} ion, which is unrelated to light emission.

The photoprotein aequorin obtained from the jellyfish *Aequorea* emits light when Ca^{2+} is added (1). The luminescence takes place through an intramolecular reaction that is triggered by the binding of Ca^{2+} ions to the photoprotein (2,3). Aequorin has three Ca^{2+}-binding sites of E-F hand structure on the basis of its amino acid sequence (4). It was recently reported, however, that the luminescence of this photoprotein requires only two Ca^{2+} ions per photoprotein molecule by the luminometric titration of natural aequorin (5), resulting in a renewed question concerning the number of the Ca^{2+}-binding sites of aequorin.

Natural aequorin is heterogeneous, being a mixture of a number of iso-forms (6), and the molecular weights (20,000 $\sim$ 22,000) of its iso-forms have never been measured with a high level of precision. In contrast, recombinant aequorin is homogeneous and has a well defined molecular composition (4,7). Thus, recombinant aequorin is clearly more suitable than natural aequorin for the studies that require a high level of precision. In spite of the fact that recombinant aequorin is not completely identical with any iso-form of natural aequorin in certain molecular properties (8), it appears that all kinds of aequorin are identical in the fundamental properties relating to the function of Ca^{2+}-triggered light emission.

The present study was carried out in order to clarify the ambiguity concerning the stoichiometry of binding between aequorin and Ca^{2+} ions.

MATERIALS AND METHODS

Recombinant apoaequorin. The protein consisting of 191 amino acid residues was prepared using a bacterial secretion system and purified by anion exchange chromatography to a purity higher than 95%, as previously described (7,9). The product was further purified by reversed phase HPLC on a C4 column (1 × 15 cm; Nakarai Chemicals, Kyoto, Japan) with a linear gradient of 20–60% acetonitrile in the presence of 0.1% trifluoroacetic acid, then the eluted apoaequorin was freeze-dried. The contaminating Ca^{2+} in this purified protein was less than 2% by molar basis according to the results of inductively coupled plasma atomic emission spectroscopy (10) performed on Model UOP-1, Mark-II (Kyotokoken, Kyoto, Japan).

Recombinant aequorin. A sample of recombinant apoaequorin before the HPLC purification was converted into recombinant aequorin, and purified by two steps of chromatography, as described previously (8).

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Molecular weights of the proteins. In the present study, the molecular weights of recombinant apoaequorin and recombinant aequorin are assumed to be 21,600 and 22,050, respectively (7).

Estimation of the amount of proteins. The amounts of recombinant apoaequorin and recombinant aequorin were estimated from the $A_{1cm,280}$ values of the proteins that were determined as follows:

Recombinant apoaequorin: About 10 mg of sample was dissolved in 4 ml of water containing a trace of ammonia, at pH 9.5, and dialyzed first with 950 ml of water for 6 hr, then with the same volume of fresh water overnight. The final pH of the dialyzate was 5.8. The dialyzed solution was first freeze-dried, then further dried over P_2O_5 in a vacuum desiccator for two days. About 2 mg of the dried sample was weighed (± 5 μg accuracy), dissolved in 5 ml of 5 mM MOPS/5 mM Tris, pH 8.0, containing 0.1 M KCl, then absorbance was measured. The $A_{1cm,280}$ value of 0.1% solution of the dialyzed recombinant apoaequorin was found to be 2.10, compared to 1.78 before dialysis. The dried material might contained up to 1% weight of NH_3 judging from the amino acid composition (4), but no correction was attempted for it in the present study.

Recombinant aequorin: The dry-weight was obtained by the previously published method on natural aequorin (6). Thus, about 2 mg of sample, dissolved in 0.4 ml of 1 mM EDTA (pH 7.0), was passed through a column of Sephadex G-25 (0.7 × 12 cm; Pharmacia) that was prepared with 50 mM ammonium acetate containing 0.1 mM EDTA, pH 7.15. After measuring the A_{280} value of the eluted protein solution, exactly 1 ml of the solution was dried and weighed. The dry weight was obtained from the gross weight by subtracting the weight of a blank from which aequorin was omitted. The dry weight was further corrected for the presence of extrinsic substances bound to the protein molecule, wherein the EDTA bound to aequorin molecules was assumed to be 0.4 mol (5) and the acid-base pairing in the protein molecule was assumed to be 85% (i.e., the presence of 16 NH_3 and 3 CH_3COOH per mol). The $A_{1cm,280}$ value of 0.1% solution of recombinant aequorin was found to be 2.97.

Luminometric titration. The experiment was carried out mostly as previously described (5). Recombinant aequorin (8–10 mg) in 3 ml of 12 mM MOPS, pH 7.2, containing 0.1 M KCl and 10 μM EDTA (prepared by means of Sephadex G-25 gel filtration) was titrated with 14.82 mM $CaCl_2$ in the sample compartment of an integrating light meter; the calcium solution was added by a HPLC pump at a calibrated speed of 27.5 μl/min. The $CaCl_2$ solution was made by dissolving a known weight of $CaCO_3$ (chelometric standard, 99.95–100.05%; Alfa Aesar, Ward Hill, MA) with HCl, then diluting the solution with 5 mM MOPS buffer to the set volume (final pH 7.2).

Titration using calcium-sensitive electrode. The solution of recombinant aequorin was prepared with 12 mM MOPS, pH 7.2, containing 0.1 M KCl and 10 μM EDTA, by gel filtration (Sephadex G-25). Recombinant apoaequorin was dissolved in 5 mM Tris/5 mM MOPS, pH 8.1, containing 0.1 M KCl and 10 mM 2-mercaptoethanol, then left in a refrigerator overnight to reduce the S-S bonds that may occur in the protein. A solution (3 ml) containing approx. 10 mg of protein was titrated with 14.82 mM $CaCl_2$ by stepwise addition of the titrant (5–20 μl) under magnetic stirring. Free Ca^{2+} concentration of the solution was monitored with a mini-calcium electrode MI-600 and a micro-reference electrode MI-409F (Microelectrodes, Inc., Bedford, NH) which were connected to a pH meter in the mV mode. The additions of titrant were made at intervals of 1–2 minutes to allow the stabilization of reading at each step.

RESULTS AND DISCUSSION

A typical example of the results of luminometric titration of recombinant aequorin with Ca^{2+} is shown in Fig. 1. In this example, the required amount of 14.82 mM $CaCl_2$ to complete the light emission of recombinant aequorin (0.367 μmol) was 64.17 μl (140 s; 0.951 μmol), of which 11.69 μl (25.5 s; 0.173 μmol) was spent by the EDTA existing in the sample. Thus, the molar ratio of the Ca^{2+} that was actually required for the luminescence reaction to the recombinant aequorin is calculated to be 2.12, indicating that two Ca^{2+} ions were needed for the luminescence of recombinant aequorin. Of the amount of EDTA existed in the sample (0.173 μmol), only 0.03 μmol was the buffer component and the rest must be attributed to the EDTA that had been bound to the aequorin molecules (5,11). A high linearity of the midsection of the slope suggests that the affinities of the two binding sites for Ca^{2+} are nearly equal and that the luminescence reaction takes place only when two Ca^{2+} ions are simultaneously bound.

The results of the titration of recombinant aequorin and of recombinant apoaequorin with Ca^{2+}, using a calcium-sensitive electrode to monitor the concentration of free Ca^{2+}, are shown in Fig. 2 and Fig. 3. The latter figure includes a control run in which a known amount of EDTA (1.0 μmol) was titrated with Ca^{2+}. The titration results of buffer alone, plotted in both figures, served as the calibration line; the lines are practically straight, indicating that the increase in electrode response (mV) was proportional to the increase in the logarithm of calcium concentration. As for the titration

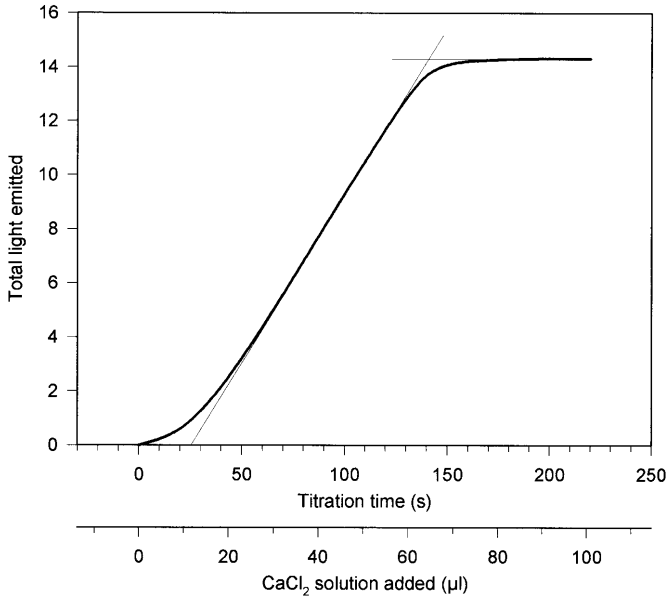

FIG. 1. Luminometric titration of recombinant aequorin (8.08 mg; 0.367 μmol) with 14.82 mM $CaCl_2$, in 3 ml of 12 mM MOPS, pH 7.2, containing 0.1 M KCl and 10 μM EDTA. Titration speed: 27.5 μl/min. Total light in arbitrary units.

curves of the proteins, the changes in the concentration of free Ca^{2+} caused by the increase of $CaCl_2$ are considerably steeper with recombinant aequorin than with recombinant apoaequorin, suggesting that the latter possibly contained the molecules of various conformations under the conditions involved.

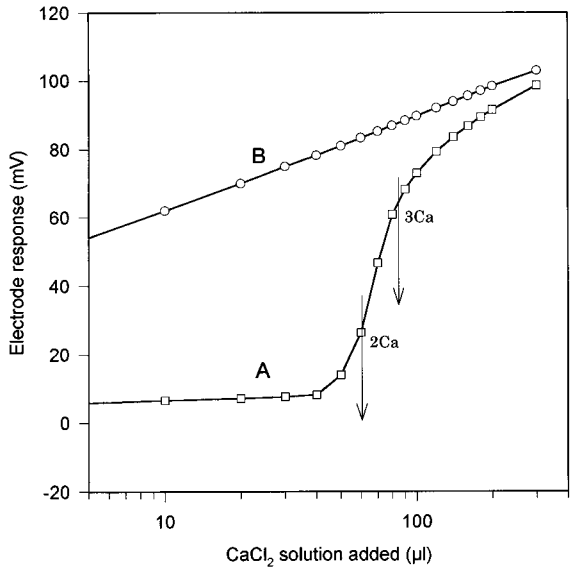

FIG. 2. Titration of recombinant aequorin (8.23 mg; 0.374 μmol) with 14.82 mM $CaCl_2$ (added stepwise), monitoring the concentration of free Ca^{2+} with a calcium-sensitive electrode, in 3 ml of 12 mM MOPS, pH 7.2, containing 0.1 M KCl and 10 μM EDTA **(A),** and a blank run without the aequorin **(B).** The vertical arrow "2Ca" indicates the amount of $CaCl_2$ equimolar to the total of the existing EDTA (approx. 0.17 μmol from the data of Fig. 1) plus twofold the amount of the aequorin; "3Ca" similarly indicates the amount of $CaCl_2$ equimolar to the total of the existing EDTA plus threefold the amount of the aequorin.

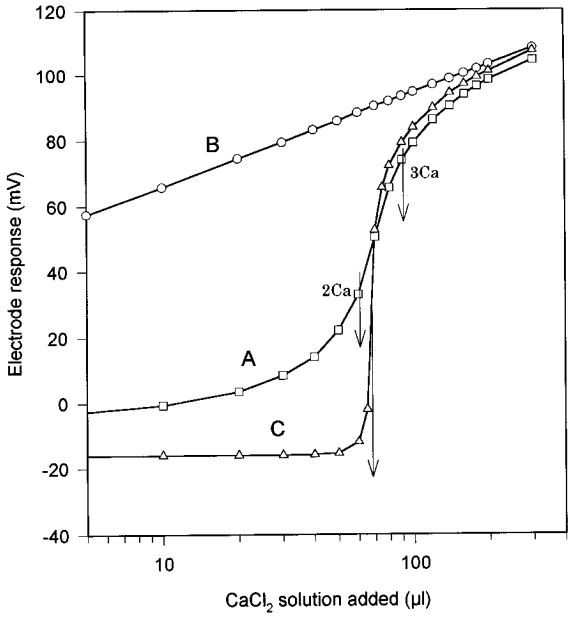

FIG. 3. Titration of recombinant apoaequorin (10.0 mg; 0.452 μmol) with 14.82 mM $CaCl_2$ (added stepwise), monitoring the concentration of free Ca^{2+} with a calcium-sensitive electrode, in 3 ml of 5 mM MOPS/5 mM Tris, pH 8.1, containing 0.1 M KCl and 10 mM 2-mercaptoethanol (EDTA absent) **(A),** a blank without the apoaequorin, **(B),** and a control with 1.0 μmol EDTA in place of the apoaequorin **(C).** The vertical arrows "2Ca" and "3Ca" indicate the amounts of $CaCl_2$ equimolar to twofold and threefold the amount of apoaequorin, respectively; the arrow without a label indicates the amount of $CaCl_2$ equimolar to the EDTA used.

In Figs. 2 and 3, the amounts of $CaCl_2$ that are equimolar to twofold and threefold the amount of the proteins (plus the amount of EDTA existing, in the case of Fig. 2) are indicated on the curves with the vertical arrows "2Ca" and "3Ca", respectively. The concentrations of free Ca^{2+} in the protein solutions at "2Ca" and "3Ca" can be obtained from the calibration line or its extrapolation, on an assumption that the ionization of $CaCl_2$ was complete. Thus, the concentration of free Ca^{2+} in the recombinant aequorin sample was found to be 2.7 μM at "2Ca", and 60 μM at "3Ca." The corresponding values of recombinant apoaequorin were 3.4 μM at "2Ca" and 91 μM at "3Ca", but these data are probably less reliable considering the possible conformational heterogeneity of the sample.

The titration curves shown in Fig. 2 and 3 clearly indicate that "2Ca" is not the end point of the titration in both cases of recombinant aequorin and recombinant apoaequorin. Therefore, the proteins must have more than two binding sites for Ca^{2+}. The "3Ca" in Fig. 2 appears to coincide with the end point of the titration, though the precise analysis of the data would be difficult due to various complicated factors, including the influence of the two Ca^{2+} ions already bound and the possible presence of several low affinity Ca^{2+}-binding sites in addition to the three binding sites of high affinities under discussion (12). The existence of such low affinity sites is noticeable in both figures, at the right end of the titration curve for the protein which is slightly lower than the calibration data.

The data presented above also show that the affinity of recombinant aequorin for the initial two Ca^{2+} ions is roughly 22 (or $10^{1.3}$) times stronger than that for the third Ca^{2+} ion, suggesting the existence of a small overlap between the binding step of the initial two Ca^{2+} ions and the binding step of the third Ca^{2+} ion. Such an overlap appears consistent with the excess value (0.12 Ca^{2+}) in the result of the luminometric titration of recombinant aequorin (2.12 Ca^{2+} per aequorin molecule).

Although the characteristics of natural aequorin in binding Ca^{2+} ions should be essentially the

same as those of recombinant aequorin, the number of Ca^{2+} ions that are needed to trigger the luminescence of natural aequorin was previously reported to be 1.98 $\sim$ 2.01 (5), whereas the corresponding data for recombinant aequorin presently obtained was 2.12. The difference is probably due to the molecular weight of natural aequorin used in the calculation (21,000) which may be slightly too low, but there is also a small possibility that natural aequorin has a weaker affinity for the third Ca^{2+} compared with recombinant aequorin.

It is the conclusion of this study that the light-emitting reaction of aequorin takes place when two of the three Ca^{2+}-binding sites of the protein are bound with Ca^{2+} ions. The Ca^{2+} affinity of the two binding sites which are necessary for the light emission is about 20 times stronger than that of the remaining binding site which is unrelated to luminescence. The role of the latter binding site, if any, remains to be elucidated.

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