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Use of Photoproteins as Intracellular Calcium Indicators

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The calcium-regulated photoproteins, of which aequorin is the best known, continue to be one of the most useful groups of intracellular Ca^{2+} indicators. They are self-contained bioluminescent systems that emit blue light in the presence of Ca^{2+} ions, can readily be purified intact, and are nontoxic when introduced into foreign cells. They have been used successfully as Ca^{2+} indicators in almost every kind of cell, but are most widely used in muscle cells because of their relative freedom from motion artifacts. Photoproteins have also been used in conjunction with microscopic image intensification to localize Ca^{2+} in cells. Their large molecular size makes them difficult to introduce into cells, but once there, they have the advantage of staying in the cytoplasm. Aequorin can be microinjected satisfactorily into single cells of almost any size, but a number of alternative methods for introducing photoproteins into cells have been developed in recent years. Disadvantages of the photoproteins for some applications include the nonlinear relation between $[Ca^{2+}]$ and light intensity, the modest speed with which they respond to sudden changes in $[Ca^{2+}]$, and the fact the Mg^{2+} antagonizes the effect of Ca^{2+}. Native photoproteins consist of a mixture of isospecies, and there are differences in Ca^{2+} sensitivity and in kinetic properties—both among photoproteins and among the isospecies of a given photoprotein. The genes for several of the isospecies of aequorin have been cloned and expressed in *E. coli*. It seems reasonable to hope that genetic engineering techniques may soon make it possible to consider using, as Ca^{2+} indicators, rare isospecies or rare photoproteins that have optimal properties for particular applications.

Introduction

It has been more than twenty years since aequorin was first used as an intracellular Ca^{2+} indicator (*1*). During that time, a great many methods for measuring intracellular Ca^{2+} concentrations have been introduced; some of those have by now dropped almost entirely from sight. The most popular of the indicators in current use is Fura-2, and that by quite a margin. Yet aequorin continues to be used by a surprisingly large and still-increasing number of investigators. My purpose here is to review the properties of aequorin that account for its durable popularity and to speculate on the prospects for improvements in those properties.

Aequorin is the best-known member of a group of substances termed calcium-activated photoproteins (*2-3*) or calcium-regulated photoproteins (*4*). I prefer the latter term for two reasons: first, because these proteins are members of the family of calcium-regulated effector proteins like calmodulin and troponin C that play such an important role in controlling a variety of cellular functions; and second, because calcium regulates the function of these proteins, but is not essential for it.

The particular function that is effected by the photoproteins is light emission, and calcium-regulated photoproteins have been identified in a variety of bioluminescent marine invertebrates, mostly coelenterates (*5,6*). These organisms control their luminescence by regulating the cytoplasmic Ca^{2+} concentration of the cells (photocytes) that contain the photoproteins, and it is testimony to the unity of biology that they regulate their cytoplasmic calcium ion concentration $[Ca^{2+}]$ over approximately the same range, and apparently by much the same mechanisms, as do higher animals (*5*). Unlike most other calcium-regulated proteins, which after binding Ca^{2+} relay signals on to other systems in order to produce physiologically detectable effects, the photoproteins serve both as detector and as effector. And unlike many other bioluminescent systems in which luciferins, luciferases, and necessary cofactors are often easily separable, the photoproteins constitute prepackaged systems, in which all of the components required for luminescence are bound tightly together and behave for most purposes as a single macromolecule. (As was indicated before, Ca^{2+} is not an essential component: the light-yielding reaction proceeds very slowly in the absence of Ca^{2+}, but is

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greatly accelerated in its presence.) This prepackaging is a very convenient feature of the photoproteins from the standpoint of their use as calcium indicators because it means that the photoprotein can be purified, concentrated, stored, and introduced into cells without any special precautions being needed to maintain proper stoichiometry of the reacting species.

Aequorin consists of a single 21-kilodalton polypeptide chain to which an imidazolopyrazinone chromophore (also referred to as coelenterazine or coelenterate luciferin) is tightly, but not covalently bound (Fig. 1*A*). The apoprotein contains three potential Ca^{2+}-binding sites. During the light-yielding reaction, the chromophore is oxidized, with the elimination of a mole of carbon dioxide and the generation of an excited state. The oxygen required for this reaction is derived from the protein, where it is bound in some as yet undetermined way: The rate of the reaction is totally independent of the availability of molecular oxygen. Because the energy for light emission is derived from the oxidative degradation of the chromophore, each molecule of photoprotein can react only once. (This constitutes a significant disadvantage of the photoproteins in some applications, but is less often a problem than is commonly supposed, because the rate of photoprotein consumption is negligibly slow in most cells under resting conditions.) Presumably the parent organism has a way of recharging the spent photoprotein, and this probably involves the replacement of the oxidized chromophore (Fig. 1*B*) with a fresh molecule of coelenterazine. This sort of recharging has been accomplished *in vitro* (*7,8*). Unlike its parent compound, the oxidized chromophore readily dissociates from the protein when the latter is stripped of Ca^{2+}. The apoprotein can be recharged by incubating it with synthetic coelenterate luciferin under Ca^{2+}-free conditions in the presence of O_2 and β-mercaptoethanol, yielding an active photoprotein that, at least superficially, seems to be indistinguishable from the native one (*7*). The next step after recharging the natural apoprotein was to charge a genetically engineered one, and this, too, has been accomplished. Several laboratories have now cloned genes for apoproteins of aequorin (*8-11*), and at least one of these has been expressed in *E. coli* and charged with synthetic coelenterazine to produce an active photoprotein (*8*).

By now calcium-regulated photoproteins have been used successfully as cytoplasmic Ca^{2+} indicators in almost every conceivable type of cell—in organisms from myxomycetes to man (*5,6*). So far, the photoproteins have been most popular among those who study muscle. (More than half of the studies published to date in which photoproteins have been used as intracellular Ca^{2+} indicators have been on muscle.) The most important reason for this is that the signals derived from the photoproteins are relatively free from motion artifacts. One might reasonably predict that indicators that alter the characteristics of an incident beam of light, as do the metallochromic dyes and fluorescent indicators, would be more subject to such artifacts than indicators which emit light against a background of total darkness. This seems, in fact, to be the case: Motion artifacts are a very serious problem in the use of the metallochromic dyes and appear to be moderately troublesome with the fluorescent indicators. If the light from an aequorin-injected muscle preparation is gathered with a large symmetrical reflector (*12*) (Fig. 2), motion artifacts seem usually to be negligible. However, it is easy to

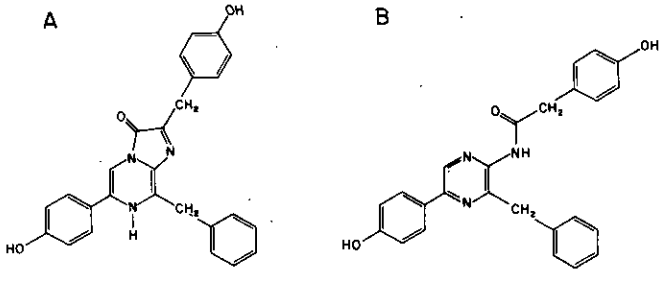

FIGURE 1. (*A*) The chromophore of aequorin (coelenterazine or coelenterate luciferin). (*B*) The oxidation product of the aequorin chromophore (coelenteramide or coelenterate oxyluciferin).

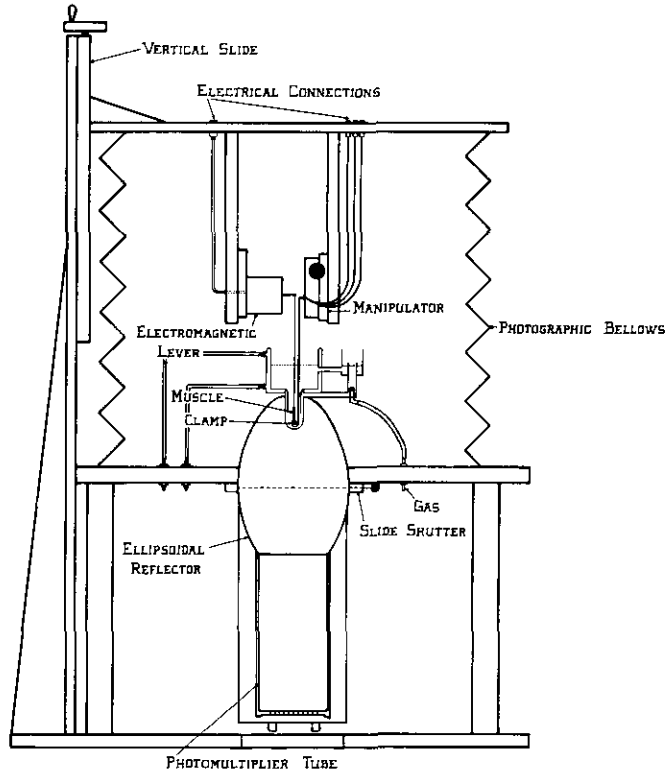

FIGURE 2. Apparatus used in our laboratory for recording light signals from aequorin-injected heart muscle. The core of the apparatus is an ellipsoidal reflector, which has one point of focus in a glass tube projecting downward from the temperature-controlled muscle bath and the other at the photocathode of the photomultiplier. The muscle is mounted at the upper focal point, and light emitted from it is equally likely to reach the photomultiplier, whatever the direction of emission. From Blinks (*12*) by permission.

imagine circumstances in which motion artifacts might readily occur—especially when light is gathered from one side, as with a microscope objective or a light guide. A related advantage of the photoproteins is that the equipment required to record the emitted light is simpler than that needed for fluorescence measurements or differential spectrophotometry. In the current enthusiasm for producing images with fluorescent indicators, a fact commonly lost sight is that with microscopic image intensification, the photoproteins can also be used very effectively to localize regions of elevated cytoplasmic [Ca^{2+}]. Aequorin was first used for this purpose as early as 1975 (*13*).

A predictable property of the photoproteins, in view of their large molecular size, is that they do not readily cross cell membranes. Both advantages and disadvantages derive from this characteristic. A major advantage, and one that is becoming more and more appreciated as experience is gained with other indicators, is that once a photoprotein has been introduced into the cytoplasm of a cell, it seems to stay there. I am not aware of any evidence that would suggest that injected photoproteins leave the cell or enter membrane-bound organelles such as the sarcoplasmic reticulum. Unfortunately, the same cannot be said for the tetracarboxylate indicators, particularly Fura-2. Of course, the negative side of this property is that one must breach the integrity of the cell membrane by one means or another in order to introduce a photoprotein into a cell. The most obvious way doing this is by microinjection, and that method has been used more than any other.

Although the technique of microinjection was initially applied primarily to very large cells, Cobbold and his collaborators have refined the methodology to the point that they have been able to record aequorin signals from a variety of different kinds of single cells of ordinary-size including fibroblasts (*14*), hepatocytes (*15*), cardiac myocytes (*16*), and adrenal chromaffin cells (*17*). The technique requires considerable skill, but Cobbold et al. (*14*) have shown that, in principle, it is possible to record useful aequorin signals from individual cells of virtually any kind. Patch pipettes can also be used to introduce photoproteins into cells (*18*); however, a potential difficulty with this approach is that the pipette must either be removed from the cell or optically shielded from the photomultiplier, since it will usually contain much more photoprotein than will the cell.

Because of the technical difficulty of microinjection, a number of investigators have turned in recent years to developing alternative methods for introducing photoproteins into cells. All of these methods depend on producing temporary leaks in the cell membrane, allowing the entry of some of the aequorin placed in the bathing solution. Nothing close to equilibration takes place with any of these methods, and the aequorin concentration achieved inside the cells is so low that signals must be recorded from populations of cells rather than from individual ones. Of course, the bathing solution must be free of Ca^{2+} during the time that the cells are exposed to the photoprotein or the indicator would be rapidly discharged. Very low extracellular divalent cation concentrations are, by themselves, capable of making cells leaky, and this may be one of the factors contributing to the effectiveness of all of the methods. However, the chemical loading technique, first described by Sutherland et al. (*19*) and developed into a practical technique by the Morgans (*20,21*), appears to depend entirely on this means of reversibly permeabilizing the cell membrane.

Several other methods involve physical disruption of the membrane and, incidentally, provide graphic illustrations of the damage produced in cell membranes by some of the supposedly innocuous handling techniques routinely used in cell biology. The first of these is centrifugation, the basis of the spin-loading method introduced by Borle et al. (*22*). These investigators found that a population of cells can be loaded with enough aequorin to record intracellular [Ca^{2+}] simply by centrifuging the cells at 200*g* for a few minutes in the presence of the photoprotein. Yamaguchi et al. (*23*) found that the amount of aequorin entering platelets during centrifugation was more than doubled if 6% dimethylsulfoxide (DMSO) was added to the medium. The scrape-loading method of McNeil and Taylor (*24*) apparently depends on breaking off small patches of membrane as the cells are scraped off the bottom of a tissue culture dish with a rubber policeman (*25*). The effectiveness of the hypoosmotic shock technique, first used in erythrocytes by Campbell and Dormer (*26*) and later applied to other cells by Snowdowne and Borle (*27*), comes as less of a surprise. Although I am not aware that the approach has been applied successfully to the introduction of photoproteins into cells, other substances have been successfully introduced into cells by the brief application of intense electrical fields (*28*). This may be a method that could be used to introduce photoproteins into certain kinds of cells.

Of the alternatives to microinjection just described, only one has been used successfully with multicellular preparations. The chemical loading method of Morgan and Morgan (*20,21*) was first used in strips of cardiac and smooth muscle but has also been applied successfully to many kinds of cells in suspension. All of the other alternative methods have, so far, been used only with suspensions of cells, though it seems quite possible that the hypoosmotic shock technique might work in multicellular preparations as well.

The Ca^{2+} concentration-effect relation (Fig. 3) of the photoproteins has a number of features that deserve comment. First, the indicator is very sensitive to changes in Ca^{2+} concentration over a broad range (more than three orders of magnitude) that includes most of the range of biological interest. Second, the curve flattens out at very low [Ca^{2+}]; that is, there is a very low level of Ca^{2+}-independent luminescence. This

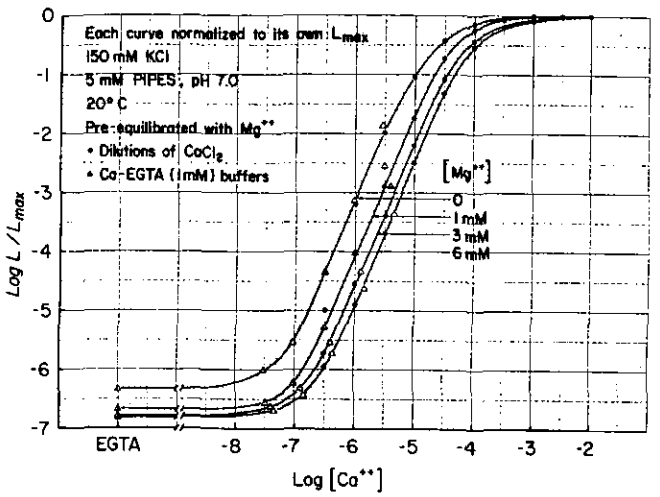

FIGURE 3. Calcium concentration-effect curves for aequorin in the presence of various concentrations of Mg^{2+}. The aequorin used for each curve was pre-equilibrated with the same concentration of Mg^{2+} present in the test solutions. (E. D. W. Moore, unpublished results.)

places a lower limit on the range of Ca^{2+} concentrations that can be detected with currently available photoproteins. The limit is sometimes, although apparently not often, reached in living cells; for those circumstances it would be desirable to have a photoprotein with a greater Ca^{2+} sensitivity than those currently available. A third important characteristic of the Ca^{2+} concentration-effect curve is its steepness. In its midrange, the curve has a slope of about 2.5 on a log-log plot, which means that light intensity changes in proportion to the 2.5^{th} power of $[Ca^{2+}]$. This is significant for both practical and theoretical reasons. From the theoretical standpoint, the most important implication is that all three of the Ca^{2+}-binding sites on the apoprotein must be involved in the regulation of luminescence. The steepness of the relation has both positive and negative practical consequences. On the positive side, it means that the indicators are very sensitive to small changes in $[Ca^{2+}]$ and that when image intensification is used to localize Ca^{2+}, there is high contrast between areas of high and low $[Ca^{2+}]$. On the negative side, it means that quantitative interpretation of the light signals is complicated when gradients of $[Ca^{2+}]$ exist, since the signals will be dominated by the regions of high $[Ca^{2+}]$, and will not represent a spatial average. This problem can be particularly troublesome when multicellular preparations are used and damaged cells dominate the signal—an argument for studying single cells or injecting the photoprotein into only one cell of a multicellular preparation whenever possible. As is shown in Fig. 3, the calcium concentration-effect curve for aequorin is significantly influenced by the Mg^{2+} concentration in the medium: Mg^{2+} acts as a competitive antagonist of Ca^{2+}. This is generally appreciated, but a less well known and highly important fact is that the binding of Mg^{2+} to aequorin is rather slow (*29–31*). This means that when curves of the sort illustrated in Figure 3 are determined, and particularly when any kind of kinetic studies are carried out, the photoprotein must be pre-equilibrated with the concentration of Mg^{2+} to be used in the test solution. When Ca^{2+} concentration-effect curves are determined, the measurements of absolute light intensity at low $[Ca^{2+}]$ are not influenced by whether or not the aequorin has been pre-equilibrated with Mg^{2+} because at very low $[Ca^{2+}]$ the light emission is measured under essentially steady-state conditions, and there is plenty of time for equilibration after the reagents are mixed. It is when $[Ca^{2+}]$ is high that pre-equilibration makes a difference. Although the total amount of light emitted by a given aliquot of aequorin is not influenced by $[Mg^{2+}]$, the peak light intensity measured when that aliquot is rapidly mixed with a saturating $[Ca^{2+}]$ is lower when the aequorin has been pre-equilibrated with Mg^{2+} than when it has not been. Thus, peak light intensity (L_{max}) is reduced by pre-equilibration, and since all values in the calibration curves are expressed as fractions of L_{max}, they are all influenced as well. Since peak light intensity is reduced by pre-equilibration with Mg^{2+} and the total amount of light emitted is not changed, the peak-to-integral ratio is reduced. This ratio is important because it enters into the calculations required for the absolute calibration of light signals recorded from cells.

In 1982, Shimomura and Shimomura reported that the properties of aequorin could be altered in significant ways by chemical modification of the protein (*32*). In particular, they reported that the slope of the Ca^{2+} concentration-effect curve could be reduced by acetylating the aequorin. This report attracted considerable attention because of the potential advantages for certain applications of a "linear" aequorin. Moore studied the properties of acetylated aequorin in considerable detail and showed that although the maximum slope of the Ca^{2+} concentration-effect curve was reduced, it was reduced from 2.5 only to about 1.9 or 2.0 (*30,31,33*). The Ca^{2+}-independent luminescence was greatly increased by acetylation, and the Ca^{2+} sensitivity was also increased somewhat (Fig. 4). However, the antagonistic effect of Mg^{2+} was also increased, with the net result that under physiological conditions the lowest detectable $[Ca^{2+}]$ was not greatly altered. Acetylated aequorin responds less rapidly than native aequorin to sudden changes in $[Ca^{2+}]$ and is very much less stable, so it is not particularly promising as a physiological Ca^{2+}-indicator. The fact that so many of the properties of aequorin were changed by acetylation is intriguing, but mechanistically not very illuminating because acetylation is such a nonspecific modification. For example, there are 15 lysine residues in apoaequorin (*34*), any or all of which might have been acetylated.

Ca^{2+} concentration-effect curves determined *in vitro* (like those shown in Fig. 3) are needed for the conversion of light signals recorded from cells into

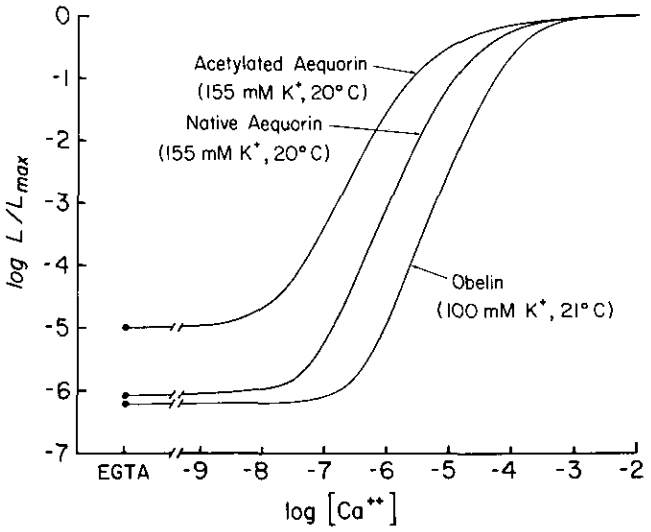

FIGURE 4. Calcium concentration-effect curves for acetylated aequorin, native aequorin, and obelin. The curve for obelin was taken from the paper of Stephenson and Sutherland (*36*), and was determined at a somewhat lower ionic strength than those determined in our laboratory. The curve for obelin would have been displaced slightly more to the right if it had been determined in 155 mM K^+. From Blinks and Moore (*30*) by permission.

absolute Ca^{2+} concentrations. Measurements of the light intensity emitted by cells or tissues can be referred to this curve only if all light intensities are expressed in the same units. In other words, all measured light intensities must be normalized for the optical conditions of measurement and for the amount of photoprotein in the system. The method for doing this introduced by Allen and Blinks (*35*) has now been adopted very widely, though it cannot yet be used satisfactorily with all tissues.

In this method, all light measurements are converted into units of fractional luminescence (L/L_{max} in Fig. 3)d by dividing them by the peak light intensity (L_{max}) that would be recorded under the optical conditions of the experiment if all of the photoprotein in the system could be exposed instantly to a saturating concentration of Ca^{2+}. *In vitro*, L_{max} is measured directly: It is the peak light intensity recorded when a standard aliquot of photoprotein is rapidly mixed with a solution containing a high [Ca^{2+}]. Because it is not possible to increase the [Ca^{2+}] inside cells rapidly enough to make a comparable measurement, one must estimate L_{max} by taking advantage of the fact that the total amount of light emitted when a quantity of aequorin is fully discharged is independent of the speed of discharge. The cell or tissue containing the photoprotein is exposed *in situ* to a detergent in order to lyse the cell membranes and expose the photoprotein to Ca^{2+} while the total amount of light emitted is integrated under the same optical conditions used for measuring light intensities earlier during the same experiment. The recorded time integral of light emission is then multiplied by a peak-to-integral ratio determined *in vitro* under appropriate conditions of temperature and [Mg^{2+}] to estimate L_{max} for the photoprotein in the cell or tissue. The detergent used most widely to lyse the cell membranes has been Triton X-100. It has been shown not to influence the quantum yield of the aequorin reaction, but it has the disadvantage of not lysing some cells (notably those of some kinds of mammalian cardiac muscle) quickly enough to permit accurate integration of the light signal. A better detergent is sorely needed for this purpose.

Very few Ca^{2+} indicators are able to follow the most rapid intracellular Ca^{2+} transients faithfully. The Ca^{2+}-selective electrodes are certainly the worst in this regard, and the metallochromic dye antipyrylazo III is the best (*6*). The photoproteins are in the middle of the pack: For example, aequorin's speed of response is probably adequate to track the Ca^{2+} transients of many kinds of cardiac muscle fairly faithfully, but certainly not those of fast skeletal muscle fibers. It is important to note, however, that there are important differences among the photoproteins in this respect: Obelin (extracted from luminescent hydroids of the genus Obelia) has been shown to respond to rapid changes in [Ca^{2+}] about three times as fast as aequorin (*36*). It will be important to determine whether still faster photoproteins exist.

Now that it has been established that genetically engineered photoproteins can be produced in the laboratory, the question arises as to what advantages might be derived from their use as calcium indicators. Economy might conceivably be one, though synthetic coelenterate luciferin is very expensive, and it is by no means clear that genetically engineered aequorin could be produced, purified, and charged more cheaply than natural aequorin. Consistency might be another, since there are small batch-to-batch variations in the properties of natural photoproteins, probably as a result of differences in the relative amounts of the various isospecies. However, this would seem not to be a compelling advantage, since under appropriate conditions aequorin is exceedingly stable, and a single batch could last the average experimenter a lifetime. I think the greatest potential advantage of applying genetic engineering techniques to producing photoproteins lies in the ability to produce useful quantities of rare photoproteins with especially desirable properties.

Not only do the various luminescent coelenterates contain different photoproteins, but at least some photoproteins (perhaps all) consist of multiple isospecies that differ from one another in practically significant ways. For example, electrofocusing shows that native aequorin consists of at least a dozen isospecies which differ, of course, in isoelectric point, but not in molecular size (*5*). At least some of these isospecies can be separated on a preparative scale by high-performance liquid chromatography (*37*). The isospecies of aequorin have not yet been characterized fully, but

evidently there are differences among them with respect to Ca^{2+}-sensitivity and kinetic properties. Native obelin also differs significantly from native aequorin in both these respects. Native obelin responds to sudden changes in $[Ca^{2+}]$ faster than native aequorin (as noted previously), but it is also considerably less sensitive to Ca^{2+} than aequorin (*36*) (Fig. 4). I am not aware of any attempts to separate isospecies of obelin.

That at least some of the isospecies of aequorin may be different gene products and not the results of posttranslational modification, is suggested by the fact that the amino acid sequence of native apoaequorin has multiple sites of microheterogeneity (*11,34*). Furthermore, once isolated, the various isospecies of aequorin run true in the purification sequence. That is, they do not break up into multiple isospecies again, as might be expected if the differences among them were the result of chemical modifications occurring *in vitro*. It seems to me that from the viewpoint of those who are primarily interested in the use of photoproteins as Ca^{2+}-indicators, the most important implication of the early successes in applying genetic engineering techniques to the production of aequorin lies primarily in the hope that it may be possible to produce other, less readily available photoproteins in the same way. The key result was the demonstration that an apoprotein produced by *E. coli* can actually be charged *in vitro* to produce an active photoprotein (*8*). (There was, of course, no guarantee *a priori* that a synthetic apoprotein with the proper amino acid sequence would have the appropriate tertiary structure for luminescence.)

I think it is reasonable to hope that once the isospecies of various photoproteins have been resolved and fully characterized and their apoproteins sequenced, we will be able to choose a few that have the most promising characteristics for various sorts of applications. We can then ask the genetic engineers to concentrate on producing them.

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