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Time-resolved fluorescence using a europium chelate of 4,7-bis-(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA)

Labeling procedures and applications in immunoassays

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We describe optimal conditions for protein labeling with a new fluorescent probe, 4,7-bis-(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA). The labeled proteins are suitable for time-resolved fluorometric applications because BCPDA forms fluorescent complexes with Eu^{3+} which exhibit very long fluorescence lifetimes. Labeling parameters such as the organic solvent used, pH, protein concentration, BCPDA excess and incubation times, were optimized accordingly. Excess BCPDA was removed by gel filtration and labeled proteins were characterized by absorbance and fluorescence measurements. The effect of labeling on the biological (binding) activity of the proteins streptavidin, avidin, monoclonal and polyclonal antibodies was also studied. It is shown that the labeled antibodies can be used for time-resolved fluoroimmunoassay applications.

Key words: Fluorescent label; Time-resolved fluorescence; Immunoassay non-isotopic; Europium; Labeled antibodies; Avidin; Streptavidin

Introduction

A number of conventional fluorescent probes are frequently used for protein labeling the most popular being fluorescein, rhodamines, dansyl chloride, anilinonaphthalenesulfonic acid, fluorescamine, *n*-(3-pyrene)-maleimide, Texas Red and phycobiliproteins (Chadwick et al., 1958; Brandtzaeg, 1976; McKinney et al., 1975; Handschin et al., 1976; Titus et al., 1982; Kronick et al., 1983). Fluorescein and to a lesser extent rhodamine are widely used in the field of non-isotopic immunoassay. These probes have been proved very successful in immunoassay applications where extreme sensitivity is not needed, such as in assays of antigens present in the sample in the micromolar to nanomolar range. Representative among the fluorescein applications in immunoassay is the fluorescence polarization immunoassay of drugs for therapeutic drug monitoring (Dandliker et al., 1973; Spencer et al., 1973). However, it is now well-established that fluorescein is not suitable for developing immunoassays with sensitivities in the picomolar range mainly because of the high background fluorescence readings encountered in the measurements (Smith et al., 1981a,b).

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The problems of conventional fluorometry in the immunoassay field have recently been reviewed (Hemmila, 1985; Diamandis, 1987) and include: separation of fluorescence emission from excitation light; Rayleigh and Raman scattering; background fluorescence from cuvettes, optics and sample; non-specific binding of reagents and fluorescence quenching. Most of these problems can be eliminated by using fluorescent lanthanide chelates as probes in combination with 'time-resolved' fluorescence. Fluorescent lanthanide chelates, and especially the Eu^{3+} chelates, exhibit a number of favorable properties. (a) They absorb radiation characteristic of the ligand, usually in the 280–350 nm region, and emit fluorescence characteristic of the metal ion, i.e., in the 615 nm region for Eu^{3+}. This is due to a unique phenomenon, the energy transfer process from the ligand to the metal ion (Kleinerman, 1969; Sinha, 1971). The Stokes shift observed is thus extraordinary ($\sim$ 290 nm for Eu^{3+}, as compared to only $\sim$ 28 nm for fluorescein). (b) The emission spectral band of Eu^{3+} fluorescence is very narrow (less than 10 nm bandwidth at 50% emission). (c) The fluorescence lifetime of these complexes is very long (10–1000 μs) compared to that of conventional fluorescence probes (3–100 ns) (Diamandis, 1987).

The unique properties of the fluorescent lanthanide complexes can be combined so that background fluorescence can be virtually excluded as follows. (a) A narrow bandpass emission filter at 615 $\pm$ 5 nm is used for Eu^{3+}. At 615 nm, serum autofluorescence, which is primarily due to albumin bound bilirubin, is very low. (b) Unwanted fluorescence in further excluded by applying the 'time-resolved' principle. After a short, pulsed excitation, measurement of fluorescence is delayed for a specified time interval (e.g., 200 μs). During this period, any short-lived fluorescence is decayed. Specific fluorescence is then measured between a time-window (e.g., 200–600 μs). Usually, this process is repeated for several cycles to improve the accuracy of the fluorescence reading (Soini et al., 1979, 1983).

Time-resolved fluorometry with lanthanide chelates as probes has potential in many areas such as flow cytometry (Leif et al., 1977) or immunohistochemistry. However, until now, it is primarily used in the field of non-isotopic immunoassay (Soini et al., 1979, 1983; Hemmila, 1985; Diamandis, 1987). Two general approaches have been tested so far. In the Delfia immunoassay system (LKB Wallac) Eu^{3+} is used to label proteins through a covalently bound ethylenediaminetetraacetic acid (EDTA) derivative serving as Eu^{3+} carrier. Excess of the chelators naphthoyltrifluoroacetone and trioctylphosphine oxide are used to form the fluorescent complex in solution at low pH (Lovgren et al., 1985). An alternative approach is to use an organic ligand as label and excess Eu^{3+} to create the fluorescent complex on the solid phase (Wieder, 1978). The relative merits and limitations of the two approaches have been recently reviewed (Diamandis, 1987).

A Eu^{3+} chelator suitable for immunoassays with the latter system must meet the following criteria. (a) It should form relatively stable complexes with Eu^{3+}. (b) The complexes must be highly fluorescent. (c) The excitation wavelength is preferable to be close to 337 nm, the emission wavelength of a nitrogen laser. (d) The chelator should covalently react with proteins. (e) It should have minimum interaction with serum components. A new chelator, 4,7-bis-(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) which meets the above criteria has been synthesized recently (Evangelista et al., 1987). The structure of the probe is shown in Fig. 1.

In this paper we describe optimized methods of labeling of a number of proteins including avidin, streptavidin, monoclonal and polyclonal antibodies, with the new probe. The labeled reagents have been tested for developing solid-phase immunoas-

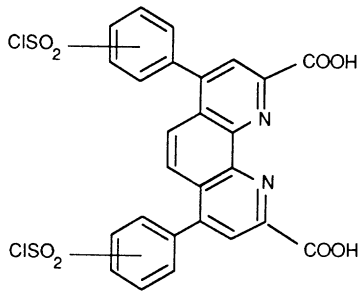

Fig. 1. The Eu^{3+} chelating reagent 4,7-bis-(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA).

says using time-resolved fluorescence spectroscopy.

Materials and methods

Instrumentation

For measurement of solid-phase fluorescence we have used the CyberFluor 615 immunoanalyzer. This instrument is a time-resolved fluorometer with a nitrogen laser as the excitation source. It is capable of measuring fluorescence at the bottom of white microtitration wells with a measuring time of 1 s/well. Data reduction is carried out automatically by the machine. A brief description of the instrument can be found elsewhere (Diamandis, 1987). For measurement of fluorescence of solutions we used the 'Arcus' time-resolved fluorometer (LKB Wallac, Turku, Finland). Spectra were recorded on the HP Model 8450A diode array spectrophotometer (Hewlett-Packard Canada, Mississauga, Ont.). High performance liquid chromatography (HPLC) was carried out using a BioSil TSK-250 size exclusion column from Bio-Rad Laboratories, Richmond, CA on a model 600 gradient system equipped with a 490 variable wavelength detector (Waters, a division of Millipore Canada, Mississauga, Ontario L4V 1M5).

Materials

Fluoroimmunoassays were performed on microtitration wells. Microfluor, white opaque wells (12-well strips) purchased from Dynatech Laboratories, Alexandria, VA 22314: 4,7-bis-(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) was synthesized as described previously (Evangelista et al., 1987). All proteins and dimethylformamide were purchased from Sigma Chemical Co., St. Louis, MO 63178. Dimethylsulfoxide, dimethylformamide, pyridine and ethanol, were reagent grade and were obtained from Fisher Scientific, Pittsburg, PA 15219. All other chemicals used were from Fisher. A monoclonal cortisol antibody was obtained from Medix Biotech, Foster City, CA 94404. Polyclonal affinity-purified goat anti-mouse IgG was obtained from Bio/Can Scientific, Mississauga, Ontario L5L 1J9. A biotinylated goat anti-human IgM antibody was purchased from Tago, Burlingame, CA 94010. Sephadex G-50, gel filtration columns, the peristaltic pump, the optical unit and fraction collector were all from Pharmacia Canada, Dorval, Quebec H9P 1H6.

Labeling of proteins with BCPDA

Generally, proteins of interest were derivatized at a final concentration of 2–5 mg/ml in 0.1 M carbonate buffer of pH 9.1, with a freshly prepared ethanolic solution of BCPDA that was added in four aliquots, at 1 min intervals, while continually vortexing the protein solution. The volume of the BCPDA solution added was such that the final percentage of ethanol in the reaction mixture did not exceed 5% of the total volume. The total amount of BCPDA added corresponded to a five-fold molar excess over the amino group concentration on the protein. The derivatization reaction was allowed to proceed for 30 min at room temperature.

Labeling of streptavidin, avidin and antibodies with BCPDA

This procedure is used when maximum biological (binding) activity rather than maximum labeling is desirable. The procedure was as above but with the following modifications. The protein concentration was 0.15 mg/ml (streptavidin and avidin) or 0.20 mg/ml (monoclonal and polyclonal antibodies). The excess of BCPDA added over the amino group concentration of the protein was two-fold (streptavidin) or 0.5-fold (avidin and antibodies). After isolating the labeled proteins or antibodies (see below) we diluted them to prepare working solutions for the assessment of their biological activity or for the assays. Working streptavidin and avidin solutions were 3 μg/ml, the working monoclonal antibody solution was 2 μg/ml and the working polyclonal antibody solution was 20 μg/ml. The diluent was a 50 mM Tris buffer of pH 7.80 containing 9 g NaCl, 10 g BSA and 0.5 g sodium azide per liter.

Isolation of BCPDA labeled protein

After the derivatization reaction was complete, the BCPDA-labeled protein was isolated by applying the reaction mixture to a Sephadex G-50 column (1.0 × 17 cm) that was equilibrated and eluted

with 50 mM NH_4HCO_3, pH 8.0. The absorbance of the effluent was monitored at 280 nm and fractions of 1.0 ml were collected. The appropriate fractions which eluted near the void volume of the column were pooled.

Quantitation of BCPDA bound to protein

In order to assess the extent of labeling, a portion of the labeled protein product was diluted to about 50–100 μg/ml and the absorbance at 325 nm was measured. The concentration of the BCPDA could be obtained by dividing the absorbance by the extinction coefficient of hydrolyzed BCPDA at 325 nm (1.5×10^4 M^{-1} cm^{-1}). The molar ratio of the BCPDA bound to the protein was obtained by dividing the BCPDA concentration by the molar concentration of the protein.

Characterization of labeled protein by fluorescence

BCPDA was hydrolyzed by incubating a weighed amount into a 0.1 M carbonate buffer of pH 9.50 at 40°C, for 2 h. This stock solution (10^{-4} M) was diluted appropriately to cover the range between 10^{-7} and 3×10^{-12} M. The dilution buffer was 10 mM Tris buffer of pH 7.80, containing 10^{-6} M Eu^{3+}. A standard curve was then constructed by plotting the fluorescence readings of each solution (Arcus fluorometer, 200 μl/well) versus the concentration of the standard BCPDA solution.

An amount of the BCPDA-labeled protein was then diluted appropriately with the same dilution buffer and the fluorescence measured with the Arcus, as above. The BCPDA concentration could then be determined from the calibration curve. The molar ratio was obtained by dividing the molar concentration of BCPDA by that of the protein.

Titration of BCPDA with Eu^{3+}

100 or 200 ml of a 10^{-8}, 10^{-7} or 10^{-6} M solution of hydrolyzed BCPDA in 10 mM Tris buffer of pH 7.80 was titrated with a 10- or 100-fold more concentrated solution of Eu^{3+}. The titration was monitored by removing 200 μl of the solution after each addition and measuring the fluorescence on the Arcus after incubation for 1 h. The total volume of Eu^{3+} solution added or removed from the titration beaker did not exceed 10 ml. Titrations were similarly performed with the labeled proteins as samples.

HPLC separation of BCPDA labeled proteins

BCPDA is a bifunctional reagent and thus, can act as a crosslinker between amino groups on the same or different protein molecules (leading to intramolecular or intermolecular linkages, respectively). In order to study the extent of intermolecular cross-linking we have subjected the labeled protein preparations to HPLC gel filtration fractionation using a Bio-Sil TSK-250 column. The mobile phase was a 50 mM Na_2SO_4 and 25 mM Na_2HPO_4 solution of pH 6.8. The flow rate was 1 ml/min and the absorbance of the effluent was monitored at 280 nm. The column used can fractionate proteins with molecular weights between 1×10^3 to 3×10^5.

Cortisol standards

We have prepared aqueous cortisol standards by diluting appropriately a stock methanolic cortisol solution.

Preparation of thyroglobulin-cortisol conjugate

The procedure is a modification of the mixed anhydride method (Erlanger et al., 1959) and it was carried out as described by Elder et al. (1987) with the exception that cortisol 21-hemisuccinate was used instead of progesterone-3-*o*-carboxymethyloxime.

Coating of microtitration strips

The coating buffer was a 0.1 M carbonate solution of pH 9.5. The blocking buffer was a 50 mM sodium phosphate solution of pH 7.4 containing 9 NaCl, 1 g gelatin and 1 ml polyoxyethylenesorbitan monolaurate (Tween 20) per liter. The wash solution was a 9 g/l NaCl solution containing 0.5 ml Tween 20 and 0.5 g sodium azide per liter.

The strips were coated overnight at room temperature with 100 μl of a 6 μg/ml solution of cortisol-thyroglobulin conjugate in the coating buffer. After coating, the strips were rinsed once with the wash solution, blocked for 1 h at room temperature with 200 μl of the blocking buffer, washed twice and air-dried overnight at room

temperature. Stored in sealed plastic bags at 4°C with dessicant, they are stable for several weeks.

Assessment of biological activity of labeled proteins

(a) Streptavidin and avidin. Microtitration wells were coated and washed as described under 'coating of microtitration strips' with a biotinylated goat anti-human IgM antibody (100 μl/100 ng/well) instead of the cortisol-thyroglobulin conjugate. 100 μl of the labeled protein solution (3 μg/ml) containing 10^{-5} M $EuCl_3$ were added to the coated wells and incubated for 1 h at 37°C. After incubation, the wells were washed three times with the wash solution and dried with a stream of cold air. The fluorescence of the complex anti-human IgM-biotin-streptavidin (or avidin)-BCPDA-Eu^{3+} was measured on the 615 immunoanalyzer.

(b) Monoclonal anti-cortisol antibody. 100 μl of the labeled antibody solution (2 μg/ml) containing 10^{-5} M $EuCl_3$ were added to thyroglobulin-cortisol coated wells and incubated as above. After washing and drying the fluorescence of the complex thyroglobulin-cortisol-antibody-BCPDA-Eu^{3+} was measured on the 615 immunoanalyzer.

(c) Polyclonal goat anti-mouse IgG. The procedure as in (b) was followed but with unlabeled monoclonal mouse anti-cortisol antibody. After incubation and washing, 100 μl of the labeled polyclonal antibody solution (20 μg/ml) containing 10^{-5} M $EuCl_3$ was added. After a further 30 min incubation at 37°C, the wells were washed, dried and the fluorescence of the complex, thyroglobulin-cortisol-monoclonal antibody-polyclonal antibody-BCPDA-Eu^{3+} was measured, as above.

Monoclonal antibody cortisol assay procedure

Before initiating the assay, the cortisol-thyroglobulin coated strips were washed twice with the wash solution. 20 μl of standards were pipetted into each well and 100 μl of the BCPDA-labeled monoclonal antibody working solution containing 10^{-5} M $EuCl_3$ was added. The strips are then briefly shaken and incubated for 1 h at 37°C. The strips are washed three times with the wash solution and dried with a stream of air. Surface fluorescence of the complex, thyroglobulin-cortisol-antibody-BCPDA-Eu^{3+} was measured on the CyberFluor 615 immunoanalyzer.

Polyclonal antibody assay procedure

The procedure is as above but unlabeled monoclonal antibody was used in the first step. After incubation and washing, 100 μl of the working (20 μg/ml) labeled second antibody solution containing 10^{-5} M $EuCl_3$ was added and incubated for 30 min at 37°C. The wells were then washed and the assay completed by proceeding as above. In this case, the fluorescent complex on the dry solid phase is thyroglobulin-cortisol-antibody-second antibody-BCPDA-Eu^{3+}.

Results and discussion

The new europium chelator, BCPDA, is not readily soluble in water unless the sulfonylchloride groups are hydrolyzed. For protein labeling, we dissolved BCPDA in a small volume of anhydrous organic solvent before its addition to the protein solution. From the organic solvents tested (ethanol, dimethylformamide (two brands) dimethylsulfoxide and pyridine) the best results were obtained with absolute ethanol. Preliminary labeling with BCPDA was carried out using BSA as a model protein. From these studies we found that separation of labeled protein from unreacted BCPDA could be easily and quantitatively achieved by using gel filtration chromatography on a Sephadex G-50 column. Alternatively, excess hydrolyzed BCPDA could also be removed by exhaustive dialysis.

For the quantitation of the BCPDA conjugated to the protein, the extinction coefficients of hydrolyzed BCPDA were calculated at 292 and 325 nm (absorption maxima) and found to be (in M^{-1} cm^{-1}) 3.4×10^4 and 1.5×10^4, respectively. The amount of BCPDA conjugated could be calculated from the absorbance at 325 nm because at this wavelength the contribution of protein to the absorbance was minimal. Maximum labeling of BSA resulted in a product having a molar BCPDA : BSA ratio of approximately 40.

The optimization of the BCPDA derivatization reaction of proteins was achieved by modifying BSA under various conditions, isolating the labeled

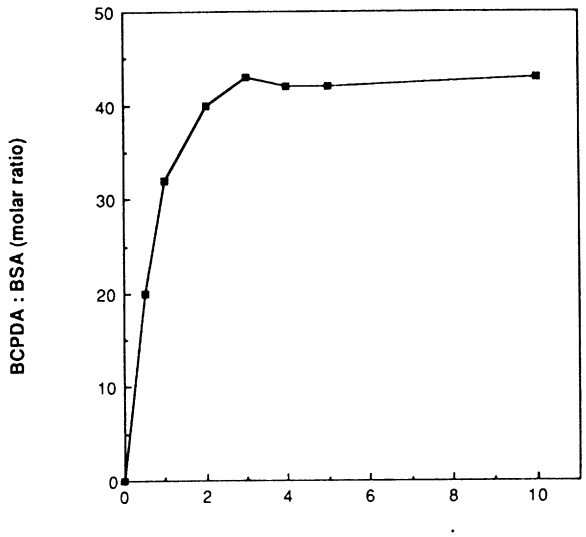

Fig. 2. BCDPA labeling of BSA. Aqueous solutions of BSA (5 mg/ml) were derivatized under standard conditions, as described in the materials and methods section, by the addition of appropriate aliquots of an ethanolic solution of BCDPA. The labeled protein was then isolated from a Sephadex G-50 column and the extent of conjugated BCPDA was assessed by absorbance at 325 nm. The molar ratio of BCPDA to BSA was then plotted against the equivalents of BCPDA (per free amino group on the protein) that was used during the labeling reaction.

protein and assessing the amount of BCPDA conjugated. The optimum labeling pH was found to be 9.1 (carbonate). Reaction was complete in less than 30 min at room temperature. The optimal protein concentration for labeling was between 2–5 mg/ml.

An additional parameter that was assessed with respect to optimizing the BCPDA labeling of proteins was the excess of BCPDA over the concentration of the amino groups used during the reaction. The amount of BCPDA added was expressed as the number of equivalents per amino group on the protein. As observed for the derivatization of BSA (Fig. 2), maximal labeling was achieved when at least three equivalents were utilized, resulting in the incorporation of about 40 BCPDA per protein molecule, on the basis of absorbance at 325 nm. In this parameter alone, the derivatization of BSA appeared to be an exception rather than the rule. For all other proteins, maximal BCPDA labeling was achieved when at least five equivalents per free amino group on the respective protein was used.

The molar ratio of BCPDA : BSA after maximal labeling, as assessed by absorbance, does not agree very closely to the total number of amino groups of BSA. This was also true for the other proteins tested, the results of which are presented in Table I. Four general mechanisms may account for this positive or negative discrepancy. (a) Because BCPDA is a bifunctional reagent, it is possible that two amino groups can be derivatized by only one BCPDA molecule. (b) The extinction coefficient may increase or decrease upon conjugation of BCPDA to the protein. (c) Some amino groups presumably are not accessible to derivatization with BCPDA. (d) Groups other than the amino groups can be derivatized with BCPDA. We did not investigate further which mechanism is operating in each case.

Theoretically, the molar ratio of BCPDA to protein could also be assessed by measuring the fluorescence of the BCPDA-Eu^{3+} complex or by titrating BCPDA with Eu^{3+}, as described in the materials and methods section. In Fig. 3 calibration curves are presented by plotting the fluorescence of the BCPDA-Eu^{3+} complex versus the BCPDA concentration. BCPDA was either hydrolyzed or conjugated to proteins at a molar ratio determined by absorbance measurements. Eu^{3+} was always in excess at 10^{-6} M. It can be seen

TABLE I

MAXIMAL LABELING OF PROTEINS WITH BCPDA [a]

Protein	Number of amino groups	Reference	Molecular weight ($\times 10^{-3}$)	Molar ratio of BCPDA to protein
SA	20	Green, 1975	60	30
AV	40	Green, 1975	67	33
BSA	59	Peters, 1975	66	40
TG	150	Mercken et al., 1985	660	175

[a] A series of proteins (*Streptomyces avidinii* streptavidin; SA, chicken egg white avidin; AV, bovine serum albumin; BSA, bovine thyroglobulin; TG, were labeled with 10 equivalents of BCPDA per free amino group on the protein, under standard conditions. The modified proteins were isolated a Sephadex G-50 column. The extent of labeling was assessed by the absorbance at 325 nm as described in the materials and methods section.

that the curves are not identical. If the curve for hydrolyzed BCPDA is taken as reference, the molar ratio of BCPDA : protein (protein is BSA, thyroglobulin, avidin) could be overestimated by a factor of about six-fold if a labeled protein solution with a BCPDA concentration of $\sim 10^{-7}$ M (as assessed by absorbance) is used as the sample. In the case of streptavidin, the overestimation was about 2–3-fold at the same BCPDA concentration level. The reason for the discrepancy is not clear. We postulate that it may be due to the formation of a 2 : 1 complex between two BCPDA molecules in close proximity on the protein and one Eu^{3+} molecule. It may also be due to a combined interaction of the protein and the attached BCPDA, with the Eu^{3+} complexed. From limited spectroscopic studies, we suggest that Eu^{3+} forms a 1 : 1 and a 1 : 2 complex with BCPDA, the second complex having a higher fluorescence quantum yield than the 1 : 1 complex (Evangelista et al., 1987) (see also below).

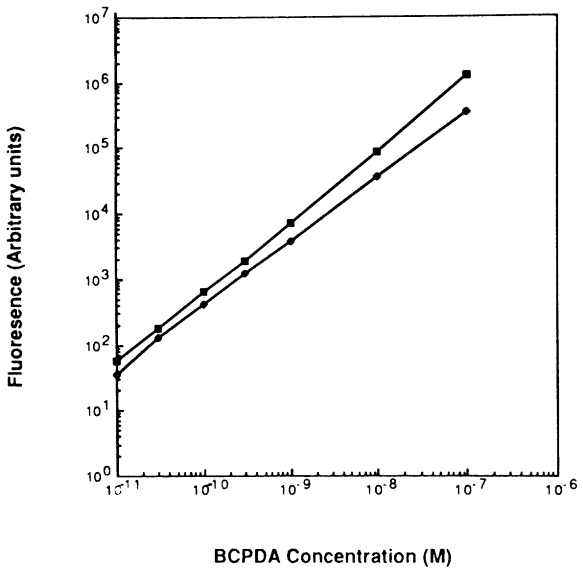

Fig. 3. Fluorescence of the Eu^{3+}-BCPDA complex in 0.01 M Tris buffer of pH 7.80. Eu^{3+} concentration was 10^{-6} M. BCPDA was either hydrolysed (●) or conjugated to thyroglobulin (■). The BCPDA concentration in the BCPDA-thyroglobulin conjugate was assessed by absorbance measurements at 325 nm. The curves for BCPDA labeled BSA and avidin were similar to that of thyroglobulin; the curve for labeled streptavidin was similar to that of hydrolyzed BCPDA up to the concentration of 10^{-8} M BCPDA.

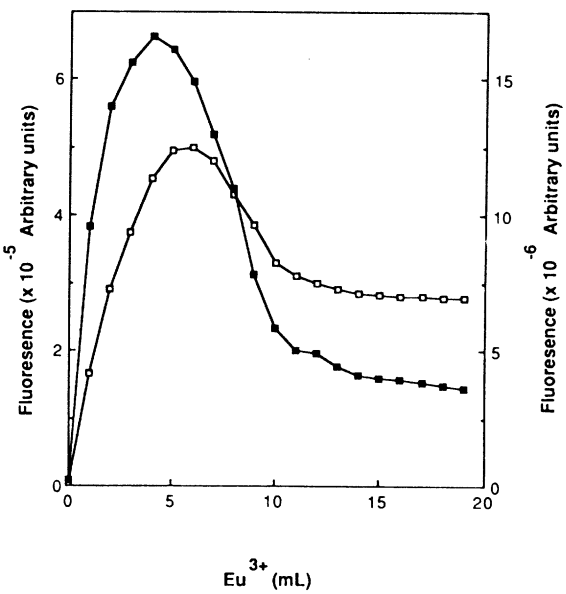

Fig. 4. Titration of 100 ml of hydrolyzed BCPDA (10^{-7} M, left fluorescence axis (□); 10^{-6} M, right axis (■) with Eu^{3+} (10^{-6} or 10^{-5} M). For more details see text.

The detection limit of measuring BCPDA with excess Eu^{3+} in solution is about 10^{-11} M.

A titration curve of BCPDA with Eu^{3+} is shown in Fig. 4. We have also titrated BCPDA (at a concentration of 10^{-7} and 10^{-8} M, 200 ml sample) conjugated to BSA, thyroglobulin, avidin and streptavidin, at molar ratios of BCPDA : protein of 40, 19, 6 (BSA), 170, 35 (thyroglobulin), 33, 7 (avidin), 30, 4 (streptavidin) (data not shown).

From the titration curve (Fig. 4) we concluded that during the initial steps of the titration, where BCPDA is in excess, complexes of the type Eu^{3+}-$(BCPDA)_n$ ($n = 2$ and probably 3) are formed. These complexes have a higher fluorescence yield than the 1 : 1 complex. After an initial peak in the BCPDA : Eu^{3+} ratio of about 2, the fluorescence drops due to the transformation of the 2 : 1 to the 1 : 1 complex. The situation with labeled proteins was more complicated (data not shown). We postulated that this was due to the following possibilities. (a) Formation of Eu^{3+}-$(BCPDA)_n$ complexes where $n = 1$, 2 or 3 having different fluorescence quantum yields. (b) The complexes could form with BCPDA molecules conjugated to the same protein molecule or different molecules. This depends on the BCPDA : protein ratio and the final

concentration of BCPDA in solution. (c) Interconversion effects from complexes with $n > 1$ to complexes with $n = 1$ during the titration. (d) Possible participation of the protein in the complexing of Eu^{3+}. (e) Quenching effects of Eu^{3+} on the fluorescence emitted. For these reasons, the equivalence points were not easily located making the assessment of protein labeling by titration unreliable.

A gel filtration HPLC column (Bio-Sil TSK-250) was utilized to assess the BCPDA labeled BSA products with respect to the amount of protein cross-linking that may have been generated as a result of the bifunctionality of BCPDA. The BCPDA labeled BSA was found to elute as a monomer with no evidence of any protein-protein crosslinking having occurred, irrespective of the number of BCPDA labels attached to the BSA molecule.

Using the optimal derivatization conditions, a series of proteins were labeled with BCPDA at an excess of five equivalents per free amino group. The resulting protein product isolated from Sephadex G-50 was assessed with respect to the amount of BCPDA incorporated, on the basis of absorbance at 325 nm (Table I). All proteins were well labeled with BCPDA. The quantitation of the incorporated BCPDA by fluorescence consistently had a value that was approximately 2 (streptavidin) or 5–6 times higher than that obtained by the absorbance of the BCPDA label. Currently, there is not enough evidence to suggest by which mechanism this phenomenon occurs, but it is clearly protein dependent.

BCPDA labeling of proteins with biological binding activity was also investigated. It was found that these proteins could tolerate the incorporation of a moderate amount of BCPDA without a significant loss in their binding activity. These proteins were labeled with varying amounts of BCPDA while ensuring that the volume of ethanol added was kept to approximately 4–5% of the final reaction volume. A small amount of organic solvent can be tolerated without proteins undergoing denaturation. The resulting protein products were isolated and the extent of BCPDA labeling was quantitated by the absorbance at 325 nm. The binding activity of the protein, as assessed by

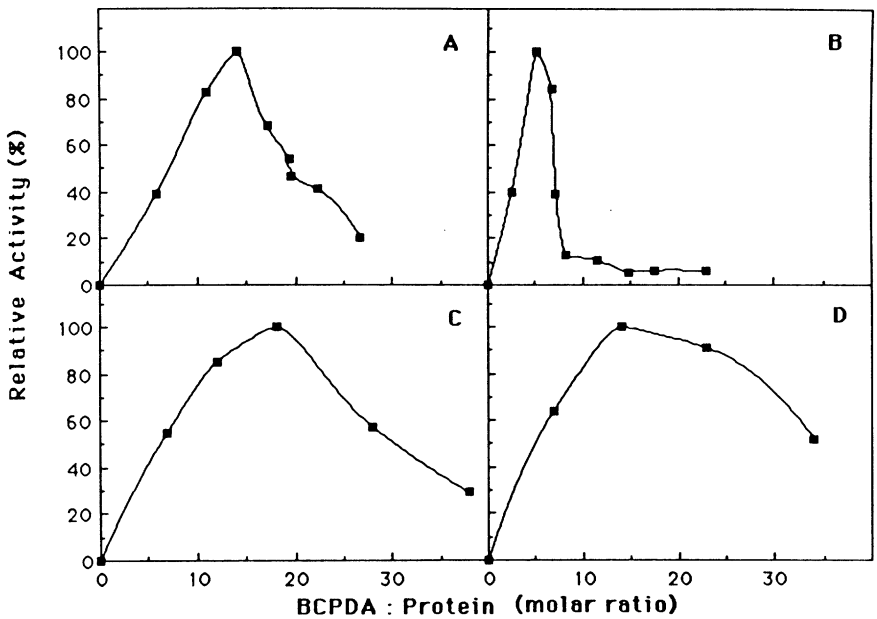

Fig. 5. BCPDA labeling of proteins and assessment of their biological binding activity. Streptavidin (*A*), avidin (*B*), monoclonal mouse anti-cortisol antibody (*C*) and polyclonal goat anti-mouse IgG (heavy and light chains) (*D*). The relative activity on the *y*-axis refers to the percentage of binding activity when the maximum binding activity observed in each case is defined as 100. The binding activity was assessed by a 'solid-phase' binding experiment as described in the materials and methods section.

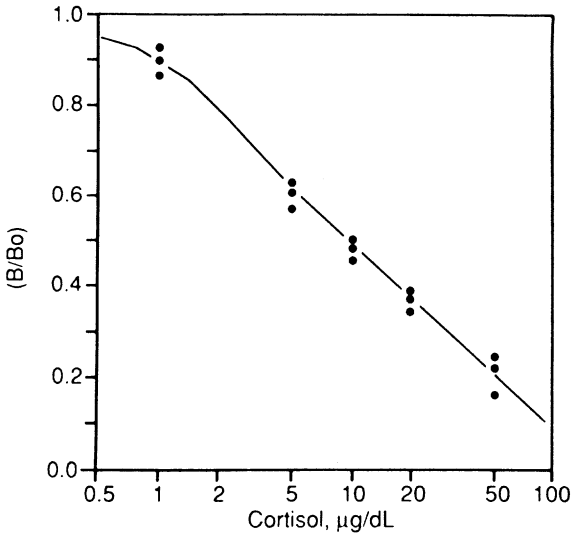

Fig. 6. Cortisol standard curve with BCPDA labeled monoclonal anti-cortisol antibody.

solid-phase binding, as described in the experimental section, was plotted against the molar ratio of the BCPDA to the protein. As observed (Fig. 5), the binding activity of the protein dramatically decreases after a critical amount of the amino groups have been derivatized. The labeling of the amino groups in the binding site of the protein would result in the decrease or loss of biological activity. The maximum number of BCPDA groups that can be incorporated while maintaining biological activity is clearly protein dependent; approximately 70, 12, 19 and 13% of the available amino groups of streptavidin, avidin, monoclonal anti-cortisol antibody and polyclonal goat anti-mouse IgG antibody, respectively, could be derivatized without significant loss of the binding activity of these proteins (Fig. 5).

The monoclonal anti-cortisol antibody that was labeled at the optimal molar ratio of 18 BCPDA per protein molecule was used to generate a standard curve for a competition type assay using aqueous cortisol standards (Fig. 6). In a similar cortisol competition assay, the BCPDA labeled goat polyclonal anti-mouse IgG antibodies (having a molar ratio of 12) were used as detection system. A calibration curve comparable to that of Fig. 6 was obtained. These curves clearly indicate that it is possible to use the labeled immunological reagents for devising new immunoassays. The application of BCPDA-labeled streptavidin for developing immunoassays, with biotinylated antibodies as complementary reagents, has recently been described elsewhere (Chan et al., 1987; Diamandis, 1987; Khosravi et al., 1987).

An interesting by-product of our studies was the observation that multi-labeled proteins (like those shown in Table I) exhibit fluorescence 3–6 times higher than that expected by their BCPDA load. This is in strong contrast to the multi-labeled proteins obtained with fluorescein as label. It is well-established that multi-labeled proteins with fluorescein as probe are not useful because of the fluorescence quenching effect (Smith et al., 1981a). Thus, even with the use of cumbersome procedures to create fluorescein-labeled proteins with incorporation of bulking agents to decrease quenching, an optimal molar ratio of 8.8 (fluorescein : protein) has been reported (Rowley et al., 1987). In our case, fluorescence quenching was not expected (and not observed) because the excitation and emission spectra of the fluorescent Eu^{3+}-BCPDA complex do not overlap at all (Diamandis, 1987; Evangelista et al., 1987). The additional 'fluorescence bonus' effect observed is another advantage of BCPDA multi-labeled proteins. In this case, multiple labeling offers a means of increasing the sensitivity of immunoassays devised with BCPDA as label.

In conclusion, we have presented optimized conditions for labeling of proteins with the new europium chelator BCPDA. The labeled proteins can be used successfully for devising new immunoassay systems employing time-resolved fluorescence spectroscopy. Practical applications using this label in the field of immunoassay, will be published in the near future. The same label is currently under investigation for applications in immunohistochemistry and flow cytometry.

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