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PREPARATION OF A PHOTOAFFINITY PROBE FOR THE VITAMIN D-DEPENDENT INTESTINAL CALCIUM BINDING PROTEIN: EVIDENCE FOR A CALCIUM DEPENDENT, SPECIFIC INTERACTION WITH INTESTINAL ALKALINE PHOSPHATASE[Δ]

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A photoaffinity probe for the vitamin D-dependent chick intestinal calcium binding protein (CaBP) has been prepared by conjugation of methyl-4-azidobenzoimidate (MABI) to lactoperoxidase-^{125}I-iodinated CaBP to yield ^{125}I-CaBP-MABI: [3 moles MABI per mole CaBP]. After incubation *in vitro* of ^{125}I-CaBP-MABI (28,000 daltons) in model systems with bovine intestinal alkaline phosphatase (AP) (67,000 daltons), a UV light-dependent crosslinking occurred to yield a conjugate with a molecular weight of 95,000 (by SDS-gel electrophoresis); no crosslinking occurred with *E. coli* alkaline phosphatase. The formation of the ^{125}I-CaBP-MABI-AP was found to occur only in the presence of calcium.

INTRODUCTION

Vitamin D_3 (1) and its hormonally active metabolite, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$](2) induce the synthesis in the chick intestine of a 28,000 molecular weight calcium binding protein (CaBP) which can comprise up to 2-3% of the soluble protein. Although the CaBP has been shown to be localized exclusively intracellularly in the intestinal epithelial cell (3,4) and the steady state level of CaBP is correlated with both the magnitude of vitamin D-stimulated calcium absorption (5) and the activity of the renal 25-hydroxyvitamin D_3-1-hydroxylase (6), its biological function is unknown. The vitamin D-dependent CaBP is also known to be widely distributed in many other tissues of birds and mammals (7) including the kidney, pancreas, brain, and placenta.

In an effort to "trap" the intracellularly localized CaBP in putative interactions with other cellular constituents, we have prepared a UV-light activatable photoaffinity probe of CaBP. Photoaffinity probes have been successfully employed in other systems such as calmodulin (8) and insulin

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receptor studies (9). The photoaffinity probe employed in our studies is a photosensitive hetero-bifunctional crosslinking reagent methyl-4-azidobenzoimidate (MABI). As a model system to evaluate the utility of the photoaffinity probe for CaBP, we have initially studied its interaction with alkaline phosphatase. Dietary vitamin D and $1,25(OH)_2D_3$ are known to increase the activity of intestinal brush border membrane-bound alkaline phosphatase (10,17,18). Also Freund and Borzemsky (11) presented preliminary evidence that CaBP from rat mucosa increased the enzymatic activity of the mucosal alkaline phosphatase under *in vitro* incubation conditions.

MATERIALS AND METHODS

Radioisotopes were purchased from New England Nuclear (Boston, MA). Methyl-4-azido-benzoimidate was purchased from Pierce Chemical (Rockford, IL). Bovine intestinal alkaline phosphatase (1140 units/mg; highly purified) and *E. coli* alkaline phosphatase (12 U/mg; electrophoretically homogeneous) were obtained from Sigma (St. Louis, MO).

Preparation of ^{125}I-CaBP-MABI

Chick intestinal vitamin D-dependent CaBP was isolated and purified by the procedure of Friedlander and Norman and then iodinated by the lactoperoxidase method of LaPorte and Storm (13). The ^{125}I-CaBP was applied to a 0.5 x 9 cm Sephadex G-100 column, and then eluted with 50 mM sodium borate, 100 mM sodium chloride, 0.2 mM $CaCl_2$, pH 9.3; a final concentration of 1.8 µM CaBP was obtained with a specific activity of 5 x 10^8 cpm/nmole. Next a series of incubations of MABI, (0.6 mg/ml) ^{125}I-CaBP, (10 µg) and nonradioactive CaBP (0.6, 0.8 and 3.3 µM) in 50 mM sodium borate, 0.2 mM $CaCl_2$, and 100 mM NaCl, pH 9.8 were conducted in the dark for 2 hr at 4°C to allow the imidoester group of the photoaffinity reagent to covalently link to the CaBP. The entire sample was then applied to a 0.5 x 9 cm Sephadex G-25 column to separate ^{125}I-CaBP-MABI from unreacted MABI and eluted with 20 mM Hepes pH 7.2. The final concentration of the ^{125}I-CaBP-MABI was 1.8 µM with a specific activity of 5.0 x 10^8 cpm/ nmole CaBP. The incorporation of azido groups into the CaBP was estimated spectrophotometrically (14) and the number of MABI molecules per CaBP was calculated to be approximately 3.0 (see Figure 1).

Photolysis and SDS Gel Electrophoresis

The ^{125}I-CaBP-MABI photoaffinity probe at concentrations varying from 50 nM to 1000 nM was mixed with either purified anti-CaBP immunoglobulins, bovine intestinal alkaline phosphatase (1140 units/mg) or *E. coli* alkaline phosphatase (12 units/mg). The incubations were done in the presence (1 mM $CaCl_2$) or absence (2 mM EGTA) of Ca^{2+}. The samples were incubated in the dark at 4°C, followed by irradiation for 2 to 3 minutes with a shortwave C-63 mineralight at 1 cm distance. Maximum activation of the aryl azide occurs at 60 seconds (14). The incubations were terminated by addition of 2.3% w/v SDS, 10% w/v glycerol, 5% v/v 2-mercaptoethanol in 0.0625 M Tris, pH 6.8 followed by boiling for 2 min. Aliquots were then run on 7-20% gradient SDS polyacrylamide gel electrophoresis SDS-PAGE (15), stained with 0.1% Coomasie blue, dried, and autoradiographed at -70°C using Kodak XR-5 X-ray film. In selected instances a Schoeffel spectrophotometer was used to densitometrically trace the autoradiogram.

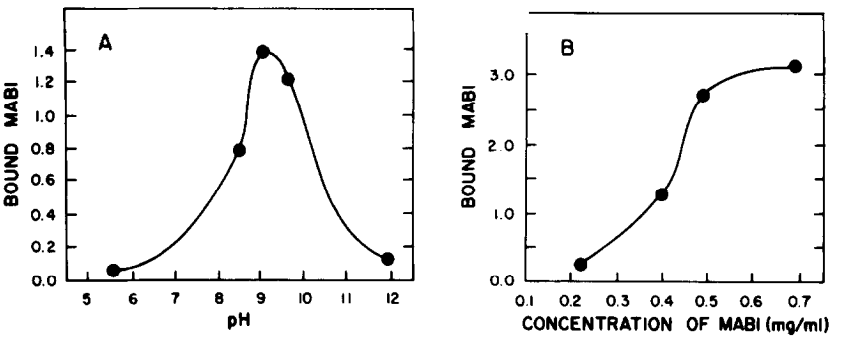

Figure 1. (A) pH dependent conjugation of the photoaffinity reagent methyl-4-azidobenzoimidate to CaBP. The MABI reagent and CaBP were incubated at concentrations of 100 nM CaBP and 0.6 mg/ml MABI reagent in 50 mM sodium borate, 100 mM NaCl, 0.2 mM $CaCl_2$ at varying pH for 60 minutes at 25°C. The number of bound MABI molecules per CaBP is plotted versus the pH of the incubation media. (B) Reagent concentration-dependent incorporation of methyl-4-azido-benzoimidate into CaBP. CaBP (100 nM) was incubated with varying concentrations of MABI for 60 minutes at 25°C.

RESULTS

Figure 1 presents data describing the pH optima (panel A) and optimum concentration of MABI (panel B) necessary to effect a defined stoichiometry of 3.0 mole MABI per mole of CaBP. This low mole ratio of photoaffinity probe to CaBP is comparable to the 1.0 for calmodulin (8) and 4-8 for concanavalin A (14) reported previously. Since CaBP has no known enzymatic activity, it is not possible to assess in detail how the covalently-linked MABI might perturb its biological function. However, as judged by chromatography of the ^{125}I-CaBP-MABI which had been incubated with radioactive calcium-45, the presence of the MABI did not interfere with its calcium binding activity (results not shown). Figure 2A presents the densitometric tracing of the autoradiogram of the SDS-PAGE of ^{125}I-CaBP-MABI incubated alone for 2 hrs followed by UV light exposure. The small light-dependent peak migrating with a molecular weight of 56,000 is consistent with the formation of a dimer of CaBP, which becomes chemically cross-linked; the dashed line tracing representing the samples not exposed to UV light indicates the purity of the ^{125}I-CaBP-MABI reagent as being at least 95%.

The crosslinking potential of the ^{125}I-CaBP-MABI reagent was assessed by incubating it with high titer monospecific rabbit antibodies (16) prepared against homogeneous chick intestinal CaBP. Clearly there is a light-dependent formation of a conjugate of ^{125}I-CaBP-MABI and the light (50,000 daltons) and heavy (87,000 daltons) of the immunoglobulins (see Figure 2B).

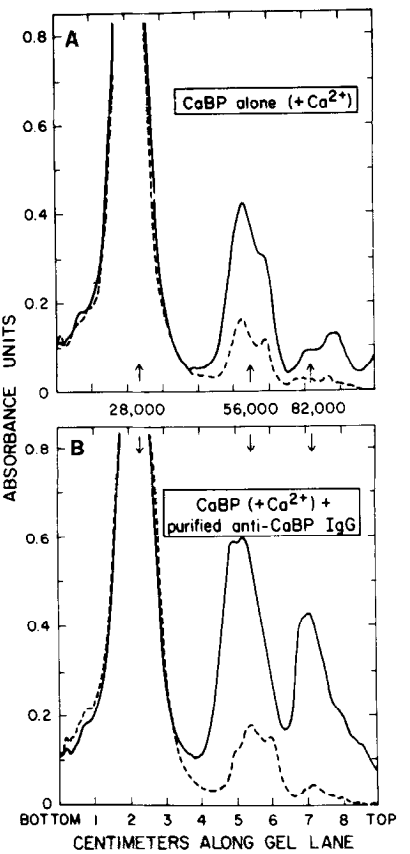

Figure 2. Densitometric tracing of an autoradiogram obtained from SDS-acrylamide gel electrophoresis of incubations of ^{125}I-CaBP-MABI alone and with anti-CaBP immunoglobulins. All samples contained 300 nM CaBP, and were incubated with the immunoglobulins for 2 h at 4°C. The top panel illustrates samples of ^{125}I-CaBP-MABI alone, while the bottom panel contains the tracing for ^{125}I-CaBP-MABI incubated with 0.2 mg/ml anti-CaBP immunoglobulin. In both tracings the solid line indicates that the sample was irradiated, while a dashed line indicates the sample was not irradiated with UV light.

The further potential of the CaBP photoaffinity probe was assessed in a model incubation system containing either highly purified bovine intestinal alkaline phosphatase or E. coli alkaline phosphatase. Figure 3A presents the actual autoradiogram of the SDS-PAGE which resulted, while Figure 3B illustrates the densitometric tracing of the autoradiogram. Clear evidence is obtained for the formation of a light-dependent conjugate (95,000 daltons) between ^{125}I-CaBP-MABI (28,000 daltons) and bovine intestinal alkaline phosphatase (BIAP) (67,000 daltons) but not E. coli alkaline phosphatase. Further, as demonstrated in Figure 4 the formation of the

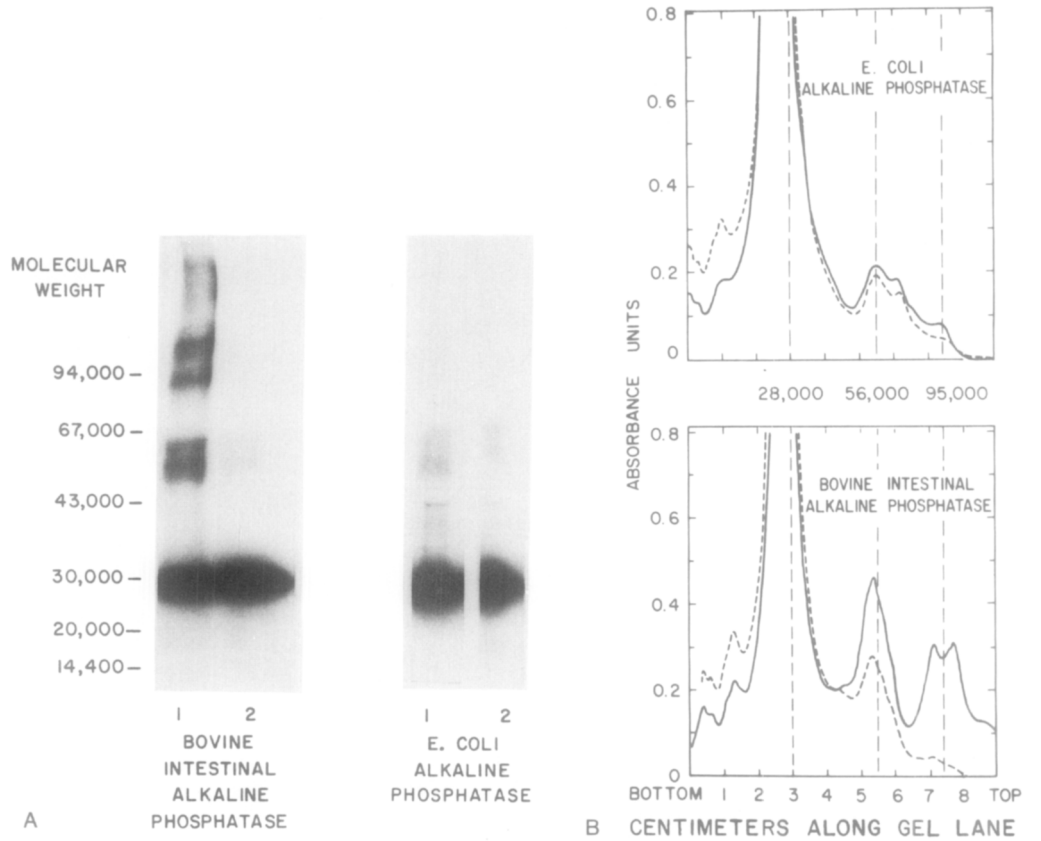

Figure 3. (A) Autoradiogram of SDS acrylamide gel electrophoresis of incubations of ^{125}I-CaBP-MABI with bovine intestinal and E. coli alkaline phosphatases. All ^{125}I-CaBP-MABI concentrations were 300 nM, and each sample contained 50 µg of alkaline phosphatase. Samples were incubated for 30 minutes at 4°C and then separated by SDS gel electrophoresis. Lane 1 contain samples which were irradiated with UV light for 2 to 3 minutes, while Lane 2 contain samples which were not irradiated. (B) Densitometric tracing from the above autoradiogram. Top panel is the tracing for incubations of the photo-affinity probe with E. coli alkaline phosphatase, while the lower panel is incubations of the ^{125}I-CaBP-MABI with bovine intestinal alkaline phosphatase. Solid lines indicate that the sample was irradiated with UV light; dashed lines indicate that the sample was not irradiated.

^{125}I-CaBP-MABI-BIAP conjugate is dependent on the presence of calcium during the incubation. When the 1 mM $CaCl_2$ was replaced by 2 mM EGTA, little or no 97,000 molecular weight conjugate could be detected after SDS-PAGE.

DISCUSSION

This report describes for the first time both the preparation of a photoaffinity probe for the vitamin D-dependent intestinal calcium binding protein as well as evidence for a specific calcium-dependent interaction of the CaBP probe with bovine intestinal alkaline phosphatase. The preparation of

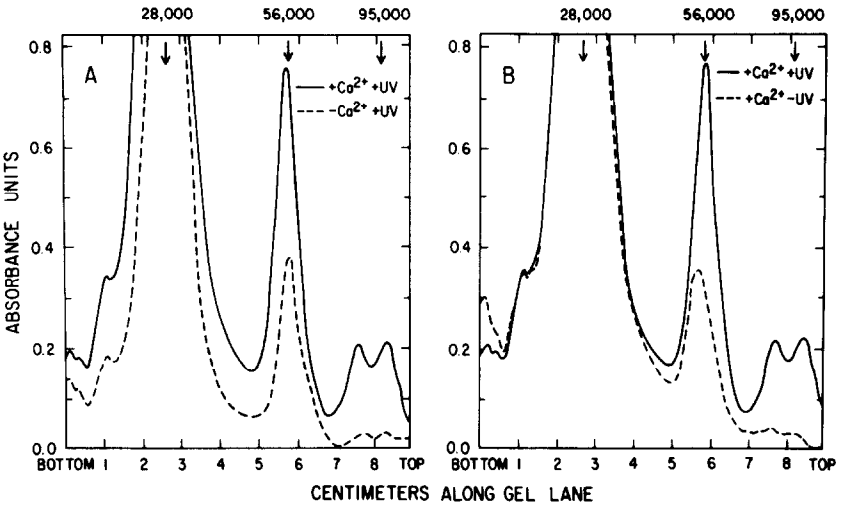

Figure 4. Demonstration of a calcium requirement for crosslinking of a photoaffinity probe of calcium binding protein (^{125}I-CaBP-MABI) to bovine intestinal alkaline phosphatase (BIAP). Panel A: ^{125}I-CaBP-MABI incubated with BIAP in the presence (solid line) or absence (dashed line) of calcium followed by UV light exposure for 3 min. Panel B: Demonstration of a UV-light dependent crosslinking of ^{125}I-CaBP-MABI to BIAP in the presence of calcium. Solid line = + UV light; dashed line = - UV light. In all incubations ^{125}I-CaBP-MABI, 300 nM was incubated with 50 µg BIAP in the presence (1 mM Ca^{2+} or absence (2 mM EGTA) of $Ca^{2+}Cl_2$ for 30 min at 4°C followed where indicated by UV-light exposure for 2-3 min. After SDS-PAGE and autoradiography, the lanes were scanned with a densitometer.

the ^{125}I-CaBP-MABI reagent was patterned on the previously described preparation of photoaffinity probes of insulin (9,14) and calmodulin (8) using the photoactivatable methyl-4-azidobenzoimidate. The functionality of the CaBP photoaffinity probe was validated by assessment of its interaction (Figure 2) with specific rabbit antisera for the CaBP.

A further test of the potential usefulness and specificity of the ^{125}I-CaBP-MABI probe was effected by comparing its interaction, in a model system, with purified bovine intestinal or E. coli alkaline phosphatase. While there are many reports (6,17-19) describing the effects of vitamin D or 1,25(OH)$_2$-D$_3$ on increasing the activity of chick, rat and bovine intestinal alkaline phosphatase and which also postulate an involvement of the alkaline phosphatase in either vitamin D-mediated intestinal calcium or phosphate transport, there are no known effects of vitamin D or its metabolites on E.coli alkaline phosphatase. As shown in Figure 3 the ^{125}I-CaBP-MABI formed a 95,000 dalton conjugate only with the intestinal alkaline phosphatase. Intriguingly, as

shown in Figure 4, this conjugate was only formed when calcium was present in the incubation medium. These results are suggestive of the possibility that Ca^{2+} may mediate conformational changes in the CaBP molecule which facilitate the interaction of the CaBP with other cellular constituents. Studies are currently in progress to assess in further detail this possibility.

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