Vol. 96, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1980 Pages 1325-1334

INTERACTION OF A RADIOLABELED CYTOKININ PHOTOAFFINITY PROBE WITH A RECEPTOR PROTEIN

Paul Keim and J. Eugene Fox

Departments of Biochemistry and Botany, University of Kansas, Lawrence, 66045

Received August 6,1980

Summary. A photoreactive analog of the cytokinin 6-benzylaminopurine was prepared by the method of Theiler et. al. (1) modified so as to include a radioactive atom in the final product, [methylene-^{14}C] 2-azido-6-benzylaminopurine. The affinity of this doubly labeled cytokinin probe for a previously described cytokinin receptor protein (2,3) is very nearly the same as for the parent cytokinin. The cytokinin probe was covalently incorporated into the receptor protein by irradiation with ultraviolet light, and its presence there was quantitatively established by assaying for non-dialyzable ^{14}C. The labeled protein was subjected to SDS polyacrylamide gel electrophoresis and the subunits assayed for radioactivity by fluorography. Each of the four subunits of the receptor protein was labeled with ^{14}C to some extent. The data suggest that all four subunits of the protein either actively participate in the formation of the cytokinin binding site or exist in close proximity to it.

Cellular constituents which exhibit relatively high affinity, reversible and specific binding to plant growth substances of the cytokinin type have been described in a number of plant systems (e.g. 2, 4, 5). The best studied and most highly purified cytokinin binding moiety is the wheat germ protein we have designated CBF-1 which has been reported to have three (6), five (4) or more (7) subunits. Because of this disagreement concerning the number of subunits and their molecular weights and because nothing is known concerning the role of the subunits in binding cytokinins, we have sought ways to label those subunits which actively participate in the binding. Although high specific activity radiolabeled cytokinins (8, 9) have been very useful in detecting and purifying low levels of cytokinin binding moieties, they are of little help in the present study since the association is a reversible and non-covalent one which does not persist through gel chromatography. Cytokinins with photolabile groups which allow the ligand to be

Abbreviations: CBF-1 = Cytokinin binding protein isolated from wheat germ
2-azido BA = 2-azido-6-benzylaminopurine
bzl^{6}Ade = 6-benzylaminopurine

0006-291X/80/191325-10$01.00/0

covalently attached to receptor proteins by photoaffinity techniques have been described (1, 10, 11). However such photoaffinity labeling has the drawback that covalently bound ligand can be detected only by competition studies. Since the subunits of CBF-1 separated by SDS-PAGE do not retain any cytokinin binding capacity (12), what is needed for this work is a radiolabeled cytokinin with a photolabile group so that covalent binding of the growth substance to the receptor site could be followed by detection of incorporated radioactivity. To this end we report here the synthesis of such a doubly labeled cytokinin probe and its interaction with the wheat germ cytokinin binding protein.

Materials and Methods. The synthesis of 2-azido-6-benzylaminopurine was carried out by the method of Theiler et. al. (1) For the synthesis of [methylene-^{14}C] 2-azido-6-benzylaminopurine a micro-procedure was adapted from this method. In 1 ml of 50 mM phosphate buffer, pH=7.8, was dissolved 2.23 x 10^{-5} mol 7-^{14}C benzylamine, sp. act. 5.6 mCi/mM (Mallinckrodt Nuclear) together with 5.3 x 10^{-5} mol 2,6-dichloropurine (Aldrich). The reaction mixture was sealed in a glass vial and incubated at 110°C for 4 hr. The white crystalline precipitate which formed was collected, by filtration washed extensively with water at 4°C and subjected to thin layer chromatography on silica gel in n-butanol, acetic acid, water 4:1:2 v/v (upper phase). More than 98% of the radioactivity was associated with a single ultraviolet absorbing band at RF 0.91 presumed to be [methylene-^{14}C] 2-chloro-6-benzylaminopurine at a calculated yield of 88%. In all subsequent steps the reaction mixture and products were protected from light. The radiolabeled product (1.34 x 10^{-5}mol) was suspended in 1 ml of 85% hydrazine hydrate and incubated in a sealed vial at 110°C for 1 hr. The reaction mixture was taken to dryness under reduced pressure, and the product was stirred at 25°C for 96 hr in 1 ml of 10% acetic acid containing 12 mg of sodium nitrite. The reaction products were separated by reverse phase HPLC on a Whatman Partisil PXS 10/25 ODS column eluted with a 0-60% ethanol linear gradient in which the aqueous phase was 20mM acetate buffer, pH=3.5. The column had been previously calibrated with an authentic sample of 2-azido-6-benzylaminopurine and radioactive fractions having the proper retention value were collected and lyophilized. The overall yield of product presumed to be [methylene-^{14}C] 2-azido-6-benzylaminopurine was determined to be 66% on the basis of the radioactivity recovered.

For procedures involving covalent attachment of the doubly labeled cytokinin, mixtures of CBF-1 prepared as previously described (12) and the cytokinin probe were incubated in the dark for 1 hr at 4°C, then irradiated in quartz cuvettes placed 10 cm from a G.E. 15 watt germicidal lamp (G15T8). Samples were then exhaustively dialyzed against 100 mM phosphate buffer, pH=7.6 for further binding studies or precipitated and washed in cold acetone, then solubilized in buffer containing 1% sodium dodecyl sulfate for gel electrophoresis. SDS-polyacrylamide electrophoresis was carried out according to Laemmli (13) and fluorography by the method of Bonner and Laskey (14) using Kodak X-omat film. Competition binding studies were done with 6-benzylaminopurine labeled with tritium in the benzene ring, sp. act. 27 Ci/mM(9).

Results and Discussion

The affinity of 2-azido BA for CBF-1. Binding of 2-azido-6-benzylaminopurine to CBF-1 was determined by competition binding studies with [^{3}H] 6-benzylaminopurine with the aid of a gel filtration assay modified from that originally described by Hummel and Dreyer (15). The affinity (K_d=6.8 x 10^{-7}M) was calculated from a double reciprocal plot (Figure 1) of the binding data and proved to be very similar to that (K_d=6.7 x 10^{-7}M) found for 6-benzylaminopurine estimated by this technique. The cytokinin activity of 2-azido-6-benzylaminopurine has been reported to be nearly as good as

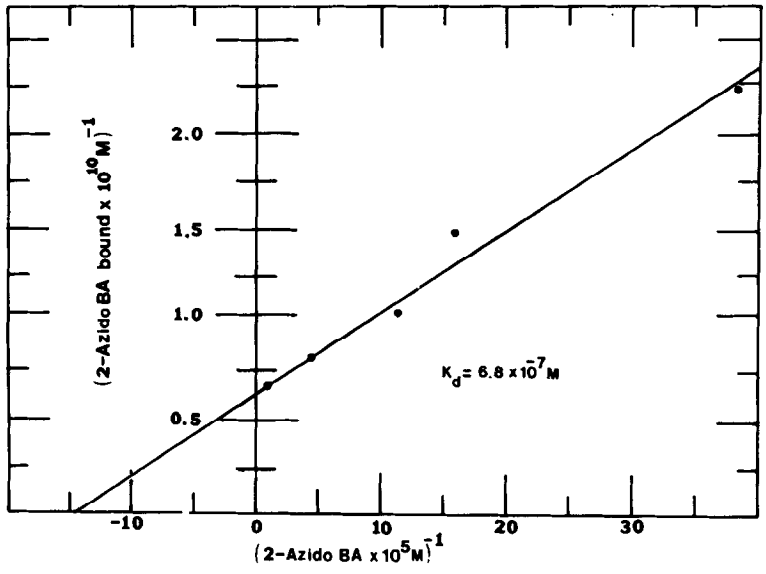

Figure 1. The affinity of 2-azido-6-benzylaminopurine for a cytokinin binding protein. In a light tight room at 4°C under a photographic safe red light a column of Sephadex G-25 0.6 cm x 4.5 cm was equilibrated in a buffer containing 1 x 10^{-9}M [^{3}H] 6-benzylaminopurine ranging from 2.6 x 10^{-7}M to 1.0 x 10^{-5}M. To the top of the column was added 25µl of a solution containing 25µg of a purified cytokinin binding protein (CBF-1) and the column was eluted with a buffer containing the same concentration of tritiated bzl^{6}Ade and unlabeled competitor as on the column. Fractions of 0.5 ml were collected and assayed for tritium by liquid scintillation spectrometry. CBF-1 was recognized by the presence of a peak of radioactivity higher than that in an equal volume of column buffer. This peak was followed by three fractions at background levels followed by a trough (fractions having lower than background radioactivity), a result expected if equilibrium has been attained (15). Binding of 2-azido-6-benzylaminopurine at various concentrations was recognized by the reduction in counts in the protein peak from [^{3}H]bzl^{6}Ade compared to a control done in the absence of competitor. 2 AzidoBA = 2-azido-6-benzylaminopurine.

bzl^{6}Ade (1) while our data indicate that the azido group at the 2 position does not interfere with binding of this cytokinin to CBF-1. The tetrazolo tautomer known to exist in a solvent dependant equilibrium with the azido form probably is minimal under the conditions employed here (1). Interaction of a closely related derivative 2-azido-N^6-(Δ^2-isopentenyl)adenine with a cytokinin binding protein from wheat germ has also been described although binding constants were not calculated (11).

In an experiment similar to that described above the photolysis products of 2-azido-6-benzylaminopurine (See Methods and Materials) were assayed for their ability to bind to CBF-1. The results indicate considerable reduction (approximately 10 fold) in the affinity of the photoproduct mixture for the protein. The cytokinin activity of the same mixture of photoproducts has been reported to be 5% of the parent compound (11) while the photoproducts of a related cytokinin 2-azido-N^6-(Δ^2-isopentenyl)adenine by contrast showed no competition with kinetin (6-furfurylaminopurine) for a binding site (11).

Covalent incorporation of [^{14}C]2-azido-6-benzylaminopurine into CBF-1.

When the doubly labeled cytokinin probe was subjected to ultraviolet irradiation in the presence of CBF-1 (Figure 2) radioactivity was incorporated into the protein. This incorporation was assumed to represent covalent binding of the molecule to a site on CBF-1 since the radioactivity could not be removed with extensive washing in acetone in which bzl^{6}Ade and its azido derivative are highly soluble. The specificity of the interaction of the ligand with CBF-1 is indicated by the lack of substantial incorporation into a control protein (ovalbumin) which has no cytokinin binding ability. Our data indicate that at the maximum ovalbumin binds 11% as much of the ligand as does CBF-1 and this binding is presumably non-specific. Under the conditions employed here, nearly maximum incorporation occurs after 60 seconds of irradiation. This finding corresponds well with our observation

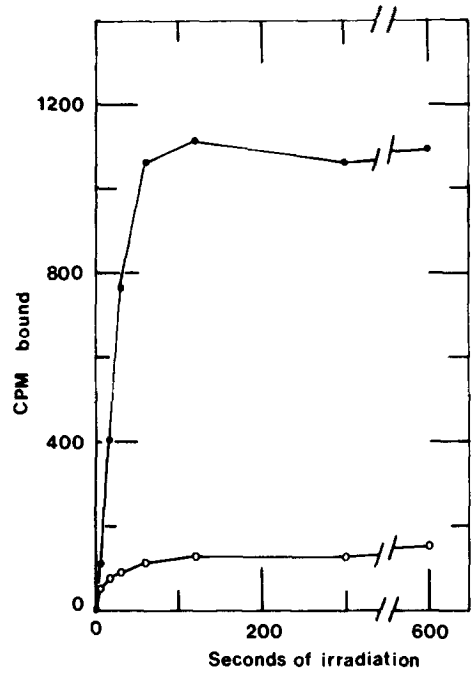

Figure 2. In a light tight room at 4°C under a photographic safe red lamp, 1 ml of a buffer containing 900µl of purified CBF-1 was incubated with 2 x 10^{-6}M [^{14}C] 2-azido-6-benzylaminopurine for 1 hr then irradiated with ultraviolet light as described in Methods and Materials. At various intervals of time 100µl aliquots were withdrawn, precipitated with 1.5 ml of acetone at 4°C and collected on nitrocellulose filters. The precipitate was washed with 10 ml of cold acetone and assayed for radioactivity by liquid scintillation spectrometry. As a control ovalbumin at the same concentration was treated in exactly the same manner.

of the kinetics of photodecay under similar conditions which canbe monitored spectroscopically by the disappearance of an ultraviolet absorbing band at 239 nm.

The addition of 6.0 x 10^{-6}M bzl^{6}Ade to a 100 µl photolysis reaction mixture containing the same concentration of [^{14}C] 2-azido-6-benzylaminopurine and 90µg of purified CBF-1 reduced by about 40% the covalent incorporation of radiolabeled ligand. That this effect is due to competition for the binding site and not to shielding resulting from the ultraviolet absorbance of bzl^{6}Ade is shown by a control study with tryptophane at a concentration (1.8 x 10^{-3}M) which provides the same absorbance at 254 nm as the lower level of bzl^{6}Ade. Here the reduction in incorporation was only 14% even though about 300 fold

more tryptophane molecules were present in the competition study. That the site itself is not damaged by the ultraviolet irradiation used in this procedure was shown in a control study in which CBF-1 was irradiated for 5 minutes under the same conditions employed in photo-labeling. Cytokinin binding after this treatment was essentially unchanged.

Inhibition of reversible binding by photoaffinity labeling. The availability of bzl^{6}Ade labeled with tritium at a high specific activity (9) made it possible to examine the binding kinetics of photoaffinity labeled CBF-1. Samples of CBF-1 which had been subjected to photoaffinity labeling in the presence of various concentrations of [^{14}C] 2-azido-6-benzylaminopurine were compared for their ability to bind 1 x 10^{-9}M [^{3}H] bzl^{6}Ade. The results (Table I) indicate that even when 0.95 mol of the photoaffinity ligand were bound/mol of protein, subsequent reversible binding of bzl^{6}Ade was reduced only 28%.

In order to investigate this surprising result more closely, samples of CBF-1 photoaffinity labeled at 0.95 mol ligand/mol protein were subjected to equilibrium dialysis binding studies (2) at a variety of

Table I

Inhibition of Cytokinin Binding in CBF-1 Photoaffinity Labeled to Different Degrees[1].

mol 2-azido BA covalently incorporated/mol CBF-1	% inhibition of equilibrium binding of bzl^{6}Ade compared to control CBF-1
.05	5
.2	10
.35	15
.63	20
.95	28

1. The cytokinin binding protein CBF-1 was subjected to photoaffinity labeling (see legend to Figure 2) in 1 ml of buffer containing 900µg of CBF-1 and concentrations of [^{14}C] 2-azido-6-benzylaminopurine ranging from 1 x 10^{-8}M to 2 x 10^{-6}M. After labeling, 100µl aliquots were withdrawn, precipitated with 1.5 ml of acetone at 4°C, washed with 10 ml of cold acetone and assayed for radioactivity in order to determine the extent of covalent labeling. The remainder of the sample was subjected to equilibrium dialysis binding against 1 x 10^{-9}M [^{3}H] bzl^{6}Ade and the reversible binding obtained compared with a control sample of CBF-1 which had been carried through the photoaffinity labeling procedure in the absence of the ligand.

concentrations of [^{3}H] bzl^{6}Ade and the results analyzed by the method of Scatchard (17). The data (Figure 3) show unexpectedly that photoaffinity labeling induces a perturbation in the apparent affinity of the protein for bzl^{6}Ade. Scatchard plots of this type have been interpreted in a variety of ways but a common explanation for this result is the presence of a high affinity site together with one or more low affinity binding sites. Indeed tangents can be drawn (Figure 3) which would indicate about a 50% reduction in the availability of the high affinity site concommitant with the appearance of at least one low affinity site. However in our view an even more

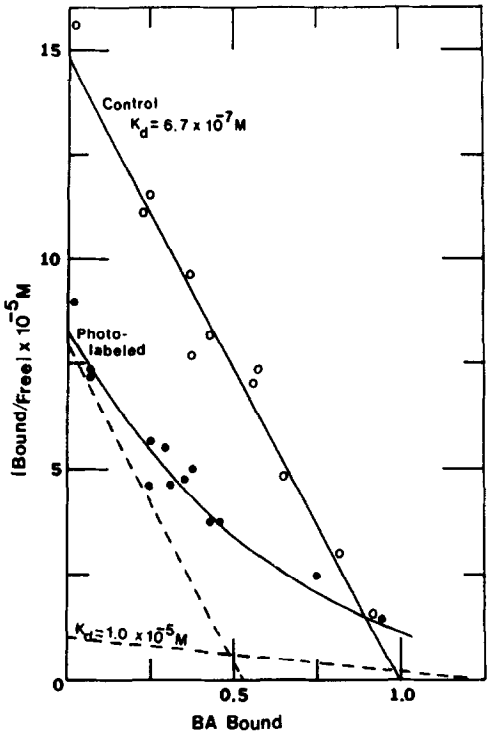

Figure 3. The cytokinin binding protein, CBF-1 was photolabeled with 2 x 10^{-6}M [^{14}C] 2-azido-6-benzylaminopurine (See legend to Figure 2) and subsequently dialyzed extensively to eliminate unbound ligand. The labeled protein was then subjected to equilibrium dialysis against concentrations of [^{3}H] bzl^{6}Ade ranging from 1 x 10^{-9}M to 1 x 10^{-5}M. Aliquots from inside and outside the dialysis bag were assayed for both ^{14}C and ^{3}H by liquid scintillation spectrometry using standard dual label counting procedures. The assay for ^{14}C indicated incorporation of the azido analog to the extent of 0.95 mole ligand/mole protein. The data for ^{3}H incorporation were plotted by the method of Scatchard (17).

likely explanation emerges from a consideration of the nature of the interaction between this photoaffinity ligand and the binding site. Substituents such as the chloro and azido moieties at the 2 position of active cytokinins do not interfere with biological activity (16) which suggests that this portion of the molecule is not intimately involved in the interaction of the ligand with the receptor site. During photoaffinity labeling, covalent cross-linking of the ligand may therefore occur with amino acids at the "edge" or periphery of the binding site and, depending upon the site of the link and the nature of the bond formed, block the binding site to a greater or lesser degree. The Scatchard plot obtained for photoaffinity labeled CBF-1 (Figure 3) may well then represent a family of curves describing the range of binding exhibited by the high affinity site whose interaction with bzl^{6}Ade is hindered to some extent depending upon the degree to which it is blocked by the photoaffinity ligand.

Subunit localization of the cytokinin binding site. In order to determine whether or not the cytokinin binding site on CBF-1 is restricted to one or more of the subunits obtained by SDS polyacrylamide gel electrophoresis, a highly purified sample of CBF-1 was subjected to photoaffinity labeling with 6×10^{-7}M [^{14}C] 2-azido-6-benzylaminopurine. This concentration was chosen to minimize non-specific binding since only about 20% of the binding sites are labeled under these conditions. This sample was subjected to SDS polyacrylamide gel electrophoresis (13) and the sub-units assayed for covalently incorporated ^{14}C by fluorography (14). The results (Figure 4) indicate that each of the four subunits which we now believe make up CBF-1 (12) has incorporated the label to at least some extent. We interpret this result to mean that all four subunits either actively participate in formation of the cytokinin binding site or exist in sufficiently close proximity to it that they are extensively labeled by the photoaffinity cytokinin probe. We favor the former interpretation since (1) cytokinin binding ability is abolished by a short incubation of CBF-1 in 6M urea and

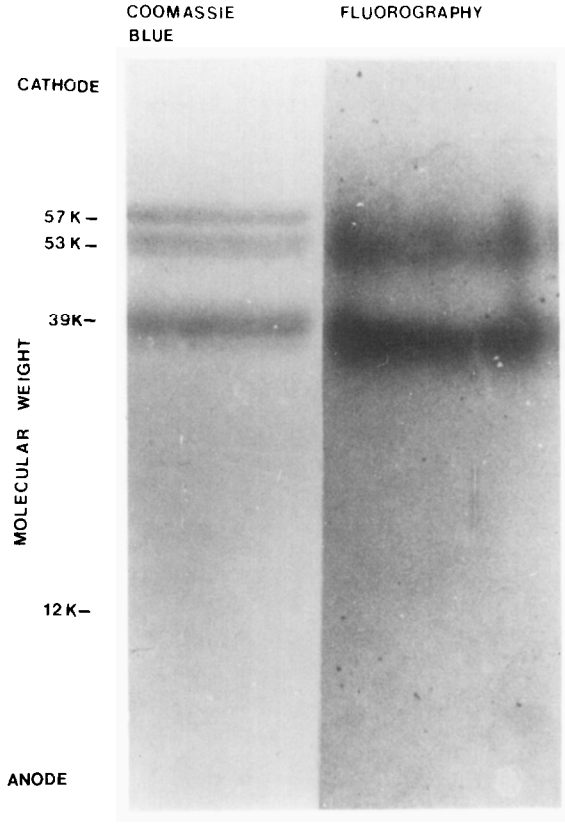

Figure 4. A highly purified sample (0.9 mg in 1.0 ml buffer) of CBF-1 was subjected to photoaffinity labeling (see legend to Figure 2) with 6×10^{-7}M [^{14}C] 2-azido-6-benzylaminopurine. The sample was then precipitated in acetone at 4°C, washed with 10 ml of cold acetone and dissolved in a solution of 1% SDS in electrophoresis buffer followed by electrophoresis on 15% acrylamide (13). Gels were either stained with coomassie blue or subjected to fluorography (14) (See Methods and Materials).

(2) subunits reisolated from gels or columns by a variety of procedures never show cytokinin binding either singly or in combinations (12).

Acknowledgements: This research was supported by grants from the National Institutes of Health (GM-22842) and the National Science Foundation (PCM76-15250).

References

1. Theiler, J. B., Leonard, N. J., Schmitz, R. Y. and Skoog, F. (1976) Plant Physiol. 58, 803-805.
2. Fox, J. E., and Erion, J. L. (1975) Biochem. Biophys. Res. Comm. 64, 694-700.
3. Fox, J. E., and Erion, J. L. (1977) In Pilet, P.E. ed. Plant Growth Regulation ppl39-146 Springer-Verlag, Berlin.
4. Polya, G. M., and Davis, A. W. (1978) Planta 139, 139-147.
5. Sussman, M. R. and Kende, H. (1978) Planta 140, 251-259.
6. Erion, J. L. and Fox, J. E. (1975) Plant Physiol. 56, 5-28.

7. Moore, F. H. III, (1979) Plant Physiol. 64, 594-599.
8. Sussman, M. R. and Firn, R. (1976) Phytochemistry 15, 153-157.
9. Fox, J. E., Erion, J. L. and McChesney, J. D. (1979) Phytochemistry 18, 1055-1056.
10. Sussman, M. R. and Kende, H. (1977) Planta 137, 91-96.
11. Mornet, R., Theiler, J. B., Leonard, N. J., Schmitz, R. Y., Moore, F. H. III,and Skoog, F. (1979) Plant Physiol. 64, 600-610.
12. Erion, J. L. and Fox, J. E. (1980) Plant Physiol (in press).
13. Laemmli, U. K. (1970) Nature, 227, 680-685.
14. Bonner, W. M. and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83-88.
15. Hummel, J. P. and Dreyer, W. J. (1962) Biochim. Biophys. Acta 63, 530-532.
16. Hecht, S. M., Leonard, N. J., Schmitz, R. Y. and Skoog, F. (1970). Phytochem. 9, 1173-1180.
17. Scatchard, G. (1949) Ann New York Acad. Sci. 51, 600-672.