Identification of the Herbicide Binding Region of the Q_B-Protein by Photoaffinity Labeling with Azidoatrazine

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Spinach thylakoids have been photoaffinity labeled with [^{14}C]azidoatrazine *in vitro* and with specific [^{3}H]amino acids *in vivo*. The radiolabeled protein has been isolated, cleaved with trypsin, and the resulting fragments have been identified by their characteristic [^{3}H]amino acid incorporation. The peptide pattern has been interpreted using the known gene sequence for the Q_B-protein of spinach thylakoids, which is the major protein labeled by both the *in vivo* and *in vitro* procedures. When analyzed by gel filtration, over 80% of the ^{14}C-activity migrated as two peaks: one as undigested protein at the void volume and the other at an apparent MW of 8.35 ± 0.76 kilodaltons. The remaining activity migrated with the included volume as free azidoatrazine. Fragments were analysed for covalent [^{14}C]azidoatrazine binding; the tryptic peptide covalently labeled by [^{14}C]azidoatrazine was found to be the fragment Pro-141 to Arg-225. This peptide is well removed from the site of a single amino acid change at Ser-264 implicated in triazine resistance, but includes a region suggested to be involved in Q_B-binding by homology with sequences of two reaction center proteins from *Rhodopseudomonas capsulata*.

Introduction

It is known that triazine herbicides inhibit photosynthetic electron transport in chloroplast thylakoid membranes via direct interference with the function of the quinone Q_B, the second stable electron acceptor at the reducing side of photosystem II (PS II) [1, 2]. From studies with the photoaffinity triazine analog, [^{14}C]azidoatrazine, it has been shown that binding occurs at a chloroplast membrane protein (the Q_B-protein) with a molecular weight (MW) of 32–34 kilodaltons (kDal) [3, 4]. Studies of highly purified PS II preparations have demonstrated that this protein is a component of the PS II reaction center complex [5]. Tryptic digestion of thylakoid membranes radiolabeled *in vivo* with [^{35}S]Met or *in vitro* with [^{14}C]azidoatrazine produces identical, sequential peptide cleavages from 34 kDal to 32, 18–19, then 16–17 kDal, with identical timecourses, indicating that the photoaffinity labeled protein is the same as the rapidly turned over 32–34 kDal protein of chloroplast thylakoids [6–8].

Recently, the sequence of the Q_B-protein derived from its nucleotide sequence in spinach has been published [9]. We have used this sequence to interpret the peptide pattern obtained by fractionation of tryptic digests of [^{14}C]azidoatrazine labeled Q_B-protein under denaturing conditions. The fragments have been identified by analysis of the patterns resulting from digestion of Q_B-protein labeled *in vivo* with several radiolabeled amino acids. Here, we report the mapping of the site of covalent attachment of azidoatrazine to an 85 amino acid fragment of the Q_B-protein.

Materials and Methods

Spinach chloroplast thylakoids were isolated as described [3], except that 5 mM $MgCl_2$ was added to all buffers, and 10 mM NaCl was omitted. *In vivo* labeling with [L-^{35}S]methionine (1240 Ci/mmol), [L-2,5-^{3}H]histidine (40 Ci/mmol), [L-3,3′-^{3}H]cystine (915 mCi/mmol; Amersham) and [L-4-^{3}H]phenylalanine (610 mCi/mmol; New England Nuclear) was performed as described [7]. In all cases, 250 µCi of radioisotope was used. Photoaffinity labeling with azido-[ring-^{14}C]atrazine (49.4 mCi/mmol; Pathfinder Laboratories) was performed by irradiating a spinach thylakoid suspension (20 mM Tricine-NaOH (pH 7.8), 5 mM $MgCl_2$, 100 mM sorbitol, 100 µg chlorophyll/ml) containing 0.59 µM

Abbreviations: Azidoatrazine, 2-azido-4-(ethylamino)-6-(isopropylamino)-*s*-triazine; PS, photosystem; Q_B, secondary quinone electron acceptor of photosystem II.

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[^{14}C]azidoatrazine with a germicidal UV lamp (American Ultraviolet; 1.5 mW/cm^2 at sample surface) at 0 °C. The sample was photolysed as a 2.5 mm deep layer in a plastic petri dish covered with Saran wrap (Dow).

Preparative electrophoresis was performed on 3 mm thick, 15 cm long, 10–15% 4 M urea gradient slab gels, with 2 cm, 4 M urea stacking gels incorporating the buffer system of Laemmli [10]. Gels were fixed in 40% aqueous methanol containing 10% acetic acid, then stained and destained as described [11]. Labeled Q_B-protein was located by slicing, dissolving (30% H_2O_2, 2% NH_4OH, 75 °C, 4 h), and assaying a 1 mm wide test strip (Amersham ACS cocktail; Packard Model 2409 Liquid Scintillation Counter). The Q_B-protein portion of the preparative gel was then excised, soaked 2 × 1 h in 50% methanol, 1 h in electroelution buffer (50 mM Tris acetate (pH 7.8), 0.1% SDS), and electroeluted (ECU-40, CBS Scientific) at 70 V, for 20 h, through the same buffer onto Spectrapor 2 dialysis membrane [12].

Electroeluants were trypsinized [13] in siliconized glass tubes (AquaSil, Pierce) for 3 h at 37 °C, in electroelution buffer containing 2% octylglucoside (Calbiochem), 3.8 mM ethylenediaminetetraacetic acid, and 13.6 µg/ml trypsin (TPCK-treated, Worthington). Gel permeation chromatography on LH-60 (Sigma) in formic acid:ethanol was performed as described [14] (see legend, Fig. 1).

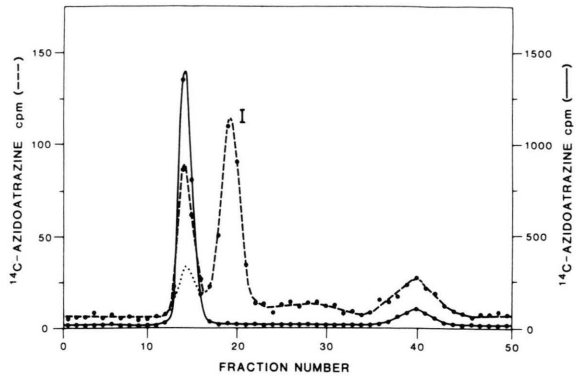

Fig. 1. Fractions obtained following gel filtration of [^{14}C]azidoatrazine labeled Q_B-protein (——) and its tryptic fragments (---) on an LH-60 column. The column (1 cm × 60 cm) was eluted with formic acid:ethanol, 1:2.8 (v/v) at 3.0 ml/h, and 1 ml fractions were collected. The samples were loaded in 1 ml of elution solvent. The void volume and included volume were measured with bovine serum albumin and Coomassie blue, respectively; molecular weights were approximated from the partition coefficients of bacteriorhodopsin proteolytic fragments [22]. The relative height of the void volume peak after 3 additions of trypsin, 6 h apart, is also shown (····).

Results and Discussion

Following trypsin digestion of the [^{14}C]azidoatrazine labeled Q_B-protein, the tryptic fragments were separated by gel filtration and fractions analysed for ^{14}C by liquid scintillation counting. The pattern of [^{14}C]azidoatrazine labeling of the Q_B-protein tryptic peptides is shown in Fig. 1. The activity eluted as three peaks: one peak at the void volume, another at an apparent molecular weight of 8.35 ± 0.76 kDal (peak I), and a minor peak at the included volume. An experiment in which untreated, [^{14}C]azidoatrazine labeled Q_B-protein was fractionated on the same column is also shown in Fig. 1. For the undigested protein, the presence of ^{14}C at the included volume shows that a small percentage (12%) of radiolabel dissociates from the Q_B-protein in the solvent system used for elution. Successive treatments with trypsin reduced the void volume peak relative to peak I. This indicates that the void volume peak observed following a single trypsin treatment is due to undigested or partially digested material. Only peak I represents a [^{14}C]azidoatrazine labeled tryptic peptide which contains no further tryptic sites.

The possible terminal tryptic fragments which can arise from the Q_B-protein are listed in Table I, along with their molecular weights and contents of four selected amino acids. The abbreviations used reflect reports [15] that translation actually starts at Met-37 in the sequence of Zurawski *et al.* [9]; for this analysis, we have used the published residue numbers for the spinach protein. The high molecular weight of the azidoatrazine labeled fragment (peak I, Fig. I) indicates that it could be T_2 or T_5 (see Table I). This preliminary identification is also supported by the results of trypsin digestion of intact thylakoids labeled either with [^{14}C]azidoatrazine *in vitro* or [^{35}S]Met *in vivo* [6–8]. Such digestion produces sequential degradation of the Q_B-protein to hydrophobic, membrane-bound fragments of 32 kDal, then 18–19 kDal, and finally 16–17 kDal, which are both methionine-rich and azidoatrazine labeled. This degradation pattern is best fit by sequential cleavage at Arg-64, then

Table I. Abbreviations, molecular weights, spans, and limited amino acid compositions of terminal tryptic fragments of the Q_B-protein.

Peptide	MW (Daltons)	First residue	Last residue	Met	Phe	His	Cys
T_{-2}	833	1	7	1	–	–	–
T_{-1}	963	9	16	–	–	–	–
T_0	1371	17	27	–	1	–	1
T_1	4007	28	64	1	3	–	–
T_2	7042	65	129	1	2	2	1
T_3	966	130	136	–	1	–	–
T_4	476	137	140	1	–	–	–
T_5	8931	141	225	5	8	4	–
T_6	1500	226	238	–	–	–	–
T_7	2189	239	257	–	2	1	–
T_8 [a]	1460	258	269	–	2	–	–
T_9	4762	270	312	1	6	1	–
T_{10}	1315	313	323	–	–	–	–
T_{11}	1287	324	334	2	–	1	–
T_{12}	1952	335	353	–	1	1	–

[a] Ser-264 is the site of the point mutation in atrazine-resistant Amaranthus [15].

Arg-238, and finally Arg-225, to give the methionine-rich fragment (17.4 kDal) composed of T_2, T_3, T_4, and T_5 [9].

A further test of the identity of the azidoatrazine-labeled fragment relies on the presence or absence of particular amino acids in the fragment. This test is performed by labeling the Q_B-protein *in vivo* with radiolabeled amino acids, and then analyzing the Q_B-protein tryptic pattern either by peak position on an LH-60 column or by relative migration of amino acid radiolabel and ^{14}C on an LH-60 column after *in vitro* co-labeling with [^{14}C]azidoatrazine. The results of single label experiments with [^{35}S]Met and [^{3}H]Phe, as well as a dual label experiment with [^{3}H]His and [^{14}C]azidoatrazine, are shown in Figs. 2A and 2B. The nearly equal mobility of the large, ^{3}H-containing peaks and the [^{14}C]azidoatrazine labeled peak I suggests that Met, Phe, and His all comigrate with [^{14}C]azidoatrazine. Only three tryptic fragments contain all three amino acids: T_2, T_5, and T_9 (see Table I). Fragment T_9 is an unlikely candidate both on the basis of molecular weight (4.76 kDal) and its absence from any reasonable fit to the sequential tryptic degradation of the Q_B-protein in intact thylakoids. Fragments T_2 and T_5 can be distinguished by a dual label experiment with [^{3}H]Cys and [^{14}C]azidoatrazine. The results of such an experiment are shown in Fig. 3, where the mobilities of the two radiolabels can clearly be distinguished. In addition, the ratio of the two radiolabels in the void volume is different from

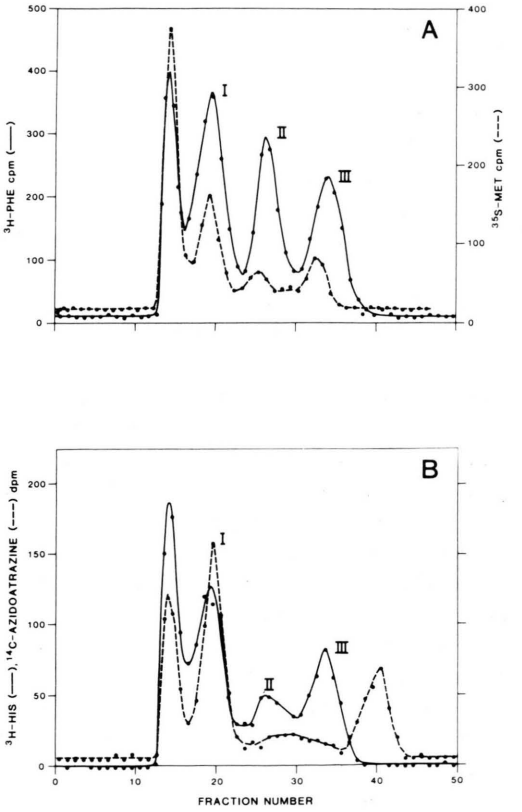

Fig. 2. Panel A: gel filtration of tryptic fragments of Q_B-protein labeled *in vivo* with [^{3}H]Phe (——) or [^{35}S]Met (---). Panel B: gel filtration of Q_B-protein dual-labeled with [^{3}H]His *in vivo* (——), and [^{14}C]azidoatrazine *in vitro* (---); dpm values are corrected for spillover. Column conditions for all samples were as in Fig. 1.

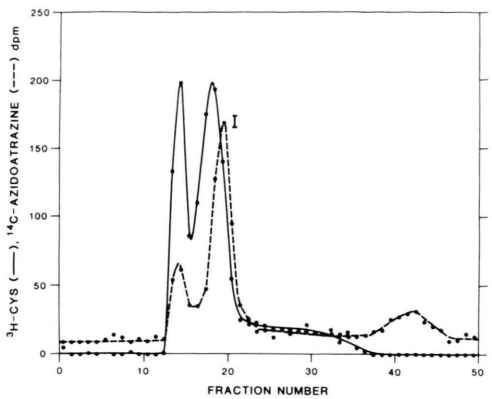

Fig. 3. Gel filtration of tryptic fragments of Q_B-protein dual-labeled with [^{3}H]Cys *in vivo* (——), and [^{14}C]azidoatrazine *in vitro* (---). The dpm values are corrected for spillover; column conditions were as in Fig. 1.

the ratio for the overlapping peaks at the position of peak I; this also shows that the two labels can migrate independently. The large amount of [^{3}H]Cys, relative to [^{14}C]azidoatrazine, in the void volume peak in Fig. 3 suggests the presence of T_2 self-dimers or mixed dimers with fragments of trypsin, which also contain Cys. Treatment of the tryptic peptides with dithiothreitol prior to gel filtration reduces the relative size of the [^{3}H]Cys void volume peak, in support of this hypothesis.

We are not certain why the [^{14}C]azidoatrazine labeled peak, assigned to T_5 (8.93 kDal), elutes at a lower apparent molecular weight than the [^{3}H]Cys labeled peak, assigned to T_2 (7.04 kDal). One explanation is that T_5 migrates anomalously, due to incomplete denaturation. It is clear from the highly variable migration of the Q_B-protein on various SDS-polyacrylamide gel systems [5] that the entire protein is difficult to denature. Anomalous migration (due to partial aggregation) of the cyanogen bromide fragments of bacteriorhodopsin has also been noted on LH-60 columns using the same solvent system [16]. Two less likely alternatives are a) cleavage of T_5 after Asp or Asn by the formic acid used to solvate the peptides [17], or b) a complete misassignment of the fragments, and [^{14}C]azidoatrazine labeling of T_9. In order to completely exclude the possibility of azidoatrazine labeling of T_9, and to clarify the assignment of the various tryptic fragments, we have calculated the numbers of residues of Met, Phe, and His in the eluted peaks and compared these to the known amino acid compositions of the tryptic fragments.

The tryptic peptides eluted from the LH-60 column fall into three molecular weight classes: a high molecular weight group (T_2 and T_5), an intermediate group (T_1 and T_9), and a low molecular weight group (remaining fragments). Assuming that the fragments elute at close to their molecular weights, we expect three eluted peaks (peaks I, II, and III, respectively), containing predictable ratios of radiolabeled amino acids. Such a pattern is demonstrated by Fig. 2. A comparison of the areas under the peaks to each other and to their predicted ratios is shown in Table II. The ranges for peak I reflect uncertainties in how much of T_2 has dimerized, and therefore migrates with the void volume. The range shown for the expected residues of Phe in peak II reflects the possibility of translation starting at Met-37 [15]. The number of amino acids equivalent to the area of peak III is assumed; other values are calculated relative to peak III. Processing of a Q_B-protein precursor [8, 18, 19] at its C-terminus [20] should not affect the Phe and His contents of T_{12}, since both amino acids occur near the N-terminus of T_{12}.

The observed and predicted peak area ratios are in close agreement (Table II). The only discrepancies are the high value observed for peak I labeled with [^{35}S]Met, and the low value observed for peak II labeled with [^{3}H]Phe. Both discrepancies can be attributed to overlaps with other large, radiolabeled peaks, which adds uncertainty to the determinations of peak areas.

Alternate schemes which exchange T_5 and T_9 are incapable of explaining the [^{35}S]Met and [^{3}H]His

Table II. Predicted and calculated contents of selected amino acids for the three major peaks observed upon LH-60 fractionation of Q_B-protein tryptic digests.

Peak	Amino acid	Contributing fragments	Residues expected	Residues calculated
I	Phe	T_2, T_5	8–10	9.2
	His	T_2, T_5	4–6	4.5
	Met	T_2, T_5	5–6	7.3
II	Phe	T_1, T_9	8–9	6.9
	His	T_9	1	0.95
	Met	T_1, T_9	2	2.2
III	Phe	T_3, T_7, T_8, T_{12}	6	6.0
	His	T_7, T_{11}, T_{12}	3	3.0
	Met	T_4, T_{11}	3	3.0

data; inclusion of T_9 in peak I with T_2 and T_5 cannot explain the large peak II in the [^{3}H]Phe data. Therefore, both qualitative and quantitative criteria indicate that the site of covalent attachment of azidoatrazine to the Q_B-protein must lie on T_5.

The location of the site of covalent attachment of azidoatrazine to the Q_B-protein is the intriguing for two reasons. First, the site is well removed in the primary amino acid sequence from the site of the single amino acid change at Ser-264 which gives rise to an *Amaranthus* biotype exhibiting greatly reduced triazine herbicide binding [15]. Either the single amino acid change affects the tertiary structure of the Q_B-protein in a subtle, indirect way, or Ser-264 lies close to the portion of T_5 labeled by azidoatrazine when the Q_B-protein is assembled into the PS II reaction center complex. Second, T_5 contains two Met-His pairings (at residues 194–195 and 198–199) suggested to be involved in Q_B binding from homology to quinone binding reaction center proteins from *Rhodospeudomonas capsulata* and several other organisms [21]. We are currently conducting experiments designed to locate precisely the site of [^{14}C]azidoatrazine covalent attachment, to further test this hypothesis.

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