Unfolding of Diphtheria Toxin

IDENTIFICATION OF HYDROPHOBIC SITES EXPOSED ON LOWERING OF pH BY PHOTOLABELING

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We report here the use of a hydrophobic photoactivatable reagent, 2-[^3H]diazofluorene (DAF), to map the hydrophobic sites exposed when the pH is lowered in diphtheria toxin (DT). The reagent binds to DT, and on photolysis with light of wavelength >350 nm, it covalently attaches itself to DT. The labeling was observed to increase considerably when the pH was lowered from 7.4 to 5.2. Although both A- and B-chains were labeled to a similar degree at pH 7.4, at lower pH (5.2), B-chain was labeled to a much higher extent. Subsequent chemical and enzymatic fragmentation of DT followed by separation indicated that the putative transmembrane domain was labeled to its maximum extent at pH 5.2, with the bulk of labeling associated with residues 340–459. Protein sequencing analysis indicated that the two buried hydrophobic helices, identified in the crystal structure and suggested to insert and span the membrane bilayer, corresponding to residues 326–347 and 358–376, are strongly labeled. The Pro-345 residue was observed to be labeled maximally at lower pH values. Finally, the DAF labeling pattern indicated that the parent structural motifs are retained at low pH, suggesting that the low pH conformation of DT corresponds to an equilibrium molten globule state.

Diphtheria toxin (DT)¹ belongs to a large class of AB toxins that act by translocation of A-chain to the cytosol and enzymatic modification of intracellular components in eukaryotic cells (1, 2). The knowledge of the crystal structure of some of these proteins has helped, but the mechanism of transfer of these proteins across the bilayer is not clear for any of these toxins. DT is composed of three domains, namely the NH₂-terminal catalytic domain C (residues 1–193), transmembrane domain T (205–378), and receptor-binding domain R (386–535) (3, 4). In DT, translocation across the bilayer occurs after receptor-mediated endocytosis to the endosomal compartment where, on acidification, the DT undergoes a conformational change to a conformation conducive to penetration of the bilayer (5–9). This initial interaction is known to induce the entry of domain C into the cytosol (10–12) where it ADP-ribosylates elongation factor-2 and thereby blocks protein synthesis leading to cell death (13). However, the acid-induced conformational change of DT which triggers the whole process is not well understood, although the evidence in favor of involvement of domain T in the initial interaction is increasing (14, 15). Thus domain T alone, on acidification, is capable of inserting into bilayers and forming channels (16–18).

We have investigated the acid-induced conformational changes in DT using the hydrophobic photoactivatable reagent 2-[^3H]diazofluorene (DAF). This reagent binds to hydrophobic sites in a protein and on photolysis generates a highly reactive carbene that inserts into neighboring groups including C-H bonds. This permits studying of the exposure of hydrophobic domains in a protein. DAF has been used in the past to study successfully the hydrophobic exposure in molten globule state of bovine α-lactalbumin (19). Conventionally, such studies have been carried out using polarity-sensitive fluorescent probes like 1-anilino-8-naphthalene sulfonic acid (ANS), which have provided very useful information. However, these probes can at best provide a general picture on the extent of hydrophobic exposure and do not yield information on domains to which the reagent binds. Hydrophobic photoactivatable reagents like DAF bind and on photolysis covalently link to the protein so that subsequent fragmentation of the cross-linked protein and peptide sequencing can in principle provide structural information on the hydrophobic sites exposed on change in environmental conditions. We report here the photolabeling of DT as a function of pH; and by analyzing the fragmented peptides we have been able to identify the exposure of hydrophobic sites, most notably in domain T. The current results also suggest that DAF labeling provides an independent method for structural characterization of protein folding intermediates like the equilibrium molten globule state.

MATERIALS AND METHODS

All reagents used were of the highest commercial grade purity available. Spectral grade water was obtained using Milli-Q Plus (Millipore Corp., Bedford, MA). Tritiated fluorenone was obtained by catalytic tritium exchange of 2-iodofluoren-9-one, at BRIT, Bombay. DAF (specific activity 535 mCi/mmol) was prepared from 2-[^3H]fluorenone according to the procedure reported earlier (20, 21). Mutated DT strain (PET.15b) WTDT in BL-21 with a single E148S mutation was a generous gift from Dr. John Collier. The strain was grown in LB/Amp medium, and DT with an NH₂-terminal hexa-His tag was isolated essentially according to the procedure of Collier and co-workers (22) except that 650 µF-chelate (Ni-IDA agarose) from Tosoh was used for Ni affinity column chromatography and Resource-Q (Amersham Pharmacia Biotech) was used for anion exchange chromatography. The purified toxin was nicked with trypsin and treated ketone-treated trypsin. The intact toxin was found to be a single band as analyzed on a 12% SDS-PAGE, according to the procedure of Laemmli (23), whereas the nicked toxin gave rise to two bands corresponding to A-chain and B-chain. The concentration of DT was estimated according to the pro-
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FIG. 1. Panel A, radioactivity (●) associated with DT on photolabeling with DAF at different pH values. •, increase in fluorescence intensity of ANS on binding to DT as a function of pH. Panel B, radioactivity associated with A-chain (○) and B-chain (●) on photolabeling of DT with DAF as a function of pH. An ethanolic solution of DAF (5.85 µCi, 10 nmol) was added to 2 ml of a 1 µM solution of DT in the appropriate buffer, then the buffer used for different pH values are given under “Materials and Methods.” The final ethanol concentration in the buffer was less than 2% (v/v). The solution was then incubated in the dark for 10 min. The samples were then photolyzed for 2 min in a Rayonet minireactor with 3,500 Å lamps. The photolabeled samples were analyzed on 12% SDS-PAGE gels, according to the procedure of Laemmli (23). The gel was then stained with Coomassie Blue and the position of bands noted, destained, cut into 2-mm slices, solubilized in hydrogen peroxide, and counted, as detailed under “Materials and Methods.” In the ANS binding experiment, 0.3 µM DT in appropriate buffer was incubated with 60 µM ANS for 10 min at room temperature. The emission spectra were recorded with the excitation wavelength set at 360 nm. The emission intensity at 480 nm was then noted.

RESULTS

DAF was incubated with DT in dark and then photolyzed with light of wavelength greater than 350 nm for 2 min. Analysis of DAF-labeled DT indicated that DAF was covalently linked to DT, and the bound radioactivity could not be dissociated from DT by solvent extraction or during SDS-PAGE. In a control experiment carried out in the dark, DAF was not found to be associated with DT, indicating that the observed labeling was photochemical in nature. The DAF-labeled DT was then analyzed by SDS-PAGE, which indicated an increase in labeling with a decrease in pH (Fig. 1A). Further analysis indicated that DAF labeled both the A-chain (0.16%) and the B-chain (0.17%) to a similar extent at pH 7.4; however, at lower pH values the labeling increased considerably for B-chain relative to A-chain. Thus at pH 5.2, B-chain labeling was observed to the A-chain. Fluorescence spectra were recorded with the emission intensity at 480 nm was then noted.

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ner similar to DAF labeling.

To obtain information on the degree of labeling of the three different functional domains in DT, namely the NH$_2$-terminal catalytic domain C (residues 1–193), transmembrane domain T (205–378), and receptor-binding domain R (386–535), we took advantage of the well spread eight methionine residues (14, 115, 178, 182, 230, 314, 339, 459) in DT. Three representative CNBr fragments, CN-3 (116–178), CN-8 (340–459), and CN-9 (460–535), corresponding to each of the functional regions, were chosen and monitored for DAF labeling. Thus DT was photolabeled with DAF at pH 7.4, and the resulting product was cleaved with CNBr and separated by SDS-PAGE. M$_r$ and NH$_2$-terminal sequence analysis of the electroblotted bands identified the three fragments. Fig. 2 indicates that all three fragments are labeled; but on lowering of the pH, the increase in labeling is considerably higher in the case of CN-8 and CN-9 relative to the CN-3 fragment. These data confirm the results obtained with A- and B-chain labeling, the latter being labeled to a greater degree at low pH values. The NH$_2$-terminus of the CN-8 fragment corresponds to two long hydrophobic helices, TH-8 and TH-9, which form a helical hairpin in the core of domain T and are covered by a layer of relatively less hydrophobic helices TH-5 to TH-7, forming a quasi-helical hairpin and finally another layer of hydro-
philic TH-1 to TH-4 helices. It has been proposed that lowering the pH causes the hydrophobic TH-8 and TH-9 to be solvent-exposed, leading to their insertion into membranes (18, 30). Thus the isolated domain T or the peptide fragment corresponding to the TH-8 and TH-9 regions can form voltage-dependent channels in planar bilayers under low pH conditions (pH 5; 16, 17, 31). The maximum increase in labeling the CN-8 fragment is in keeping with these proposals.

To glean information on the amino acid residues that undergo maximum exposure on lowering of pH and are labeled by hydrophobic reagent DAF, the three CNBr fragments mentioned above were isolated from preparative scale experiments and the peptides subjected to sequencing. The PTH-derivative analysis was then carried out, and the radioactivity associated with each cycle was determined. All experiments were minimally carried out in duplicate, and the sequencing data obtained from independent photolabeling experiments were found to be similar in reference to radioactivity associated with labeled residues. The CN-8 fragment from DT labeled at pH 7.4 and 5.2 could be both sequenced and identified. However, the radioactivity associated with each cycle of the CN-8 fragment from the pH 7.4 experiment was insignificant compared with the data obtained from the pH 5.2 experiment. The radioactivity release was observed to be below the background level after nine cycles in the pH 7.4 experiment (data not shown). In the pH 5.2 experiment extensive labeling was observed as indicated by radioactivity associated with each cycle of sequencing. The CN-3 fragment isolated from DT labeled by DAF at pH 7.4 indicated that practically no radioactivity was associated with different cycles of sequencing. The CN-3 fragment isolated from DT labeled with DAF at pH 5.2 could be sequenced up to 37 cycles, beyond which the pmol yield of the PTH-derivative was too low to make definitive assignments. The major amino acids labeled in CN-3 were Val-118, Ile-123, Val-134, Val-135, Leu-138, Phe-140, and Tyr-149 (Fig. 3). This is in keeping with similar levels of radioactivity associated with CN-3 fragment isolated from labeling at pH 5.2 and 7.4 (Fig. 2).

Finally, in an effort to study the pH-induced photolabeling of Pro-345 in more detail, we labeled DT with DAF at different pH values ranging from 7.4 to 5.2, carried out the CNBr fragmentation, and isolated the CN-8 fragments. These fragments were then subjected to peptide sequencing, and the radioactivity associated with P-345 was determined. The data given in Fig. 6 clearly indicate increased labeling of Pro-345 on lowering of pH with a sharp rise below pH 5.6.

**DISCUSSION**

The parallelism between DAF and polarity-sensitive fluorescent probes like ANS to probe hydrophobic sites in proteins opens several interesting possibilities. Thus, although both probes can provide general information on exposure of hydrophobic sites as observed in DT as a function of pH, it is the covalent linkage of DAF with the protein and the subsequent radioactive analysis of peptide fragments derived from the labeled protein which make radioactive hydrophobic photactivable reagents like DAF a useful probe. An earlier study
using another hydrophobic photoactivable reagent, 3-(trifluromethyl)-3-(m-[125I]iodophenyldiazirine, also indicated an increase in the photolabeling of DT as the pH was lowered, although no peptide sequencing data were reported in this study (33). The option to sequence the isolated DAF-labeled peptides by Edman degradation and determine the actual amino acid, which has been labeled from the radioactive profile, is what makes these types of probes a powerful structural tool. Thus a relatively much higher hydrophobic exposure of B-chain than the A-chain of DT on lowering of pH could be established easily using DAF (Fig. 1). The radioactive analysis of CNBr fragments of DAF-labeled DT indicated that the TH-8 and

FIG. 5. Radioactivity and mass release on Edman degradation of CNBr fragment CN-3 (116–178) of DAF-labeled DT at pH 5.2. The CN-3 fragment was obtained and subjected to Edman degradation and PTH-derivative release (pmol, •) and the radioactivity was recorded (cpm, ♦) as described in the legend to Fig. 3. A single peptide beginning with Glu-116 was detected. The sequence assignments could be made up to 37 cycles and are given on the top.

FIG. 6. Radioactivity and mass release on Edman degradation of CNBr fragment CN-8 (340–459) isolated from DT labeled with DAF as a function of pH. The CNBr digests were processed as described in the legend to Fig. 3, and the electroblots were sequenced for 10 cycles. The assignments for the first 10 cycles are given on the abscissa.
TH-9 hydrophobic helices are exposed maximally on lowering of pH. The TH-8 and TH-9 helices that form part of the transmembrane domain T have been implicated in membrane insertion by x-ray structural data (3, 4) and several site-directed mutagenesis studies (14, 16, 34).

The major conclusions that can be drawn from the sequencing data are as follows. 1) On lowering of pH, the CN-8 fragment of domain T is labeled most heavily followed by the CN-9 fragment belonging to domain R. Apparently as the pH is lowered the hydrophobic sites in DT are exposed, leading to binding of DAF to the most hydrophobic site, which on photolysis is manifested in terms of radioactivity associated with the peptide fragment. The CN-3 fragment belonging to domain C is labeled most poorly when the pH is lowered, suggesting that this region of the molecule undergoes minimum conformational change on lowering of pH, which is in agreement with the recent crystal structure of isolated domain C determined at pH 5 (35). 2) Because the labeling of CN-3 is significant at both pH 7.4 and 5.2 (Fig. 2), the current results indicate that domain C contains several hydrophobic sites. These results suggest that the CN-3, CN-8, and CN-9 fragments, corresponding to parts of domains C, T, and R, respectively, have several hydrophobic sites exposed at low pH and can thus be expected to interact with membranes. However, although the CN-8 and CN-9 fragments show a considerable increase in hydrophobic exposure when the pH is lowered, the CN-3 fragment does not (Fig. 2), suggesting that the pH-induced insertion process is not triggered by domain C. 3) The amino acids labeled are predominantly hydrophobic, indicating that the otherwise buried hydrophobic amino acids are exposed at low pH thereby making the toxin conducive to insertion in membranes. 4) The two buried hydrophobic helices, identified in the crystal structure and suggested to insert and span the membrane bilayer (3, 32), corresponding to residues 326–347 (TH-8) and 358–376 (TH-9) are strongly labeled. It is interesting to note that the flexible loop region (348–357) between the two helices, which is suggested to be in the lumen by a number of site-directed mutagenesis studies (14, 16, 34), is not labeled by DAF. Oh et al. (18) have reported a site-directed spin labeling study carried out with cysteine-scanning mutants from the TH-9 region of the domain T in an effort to identify the residues buried in the membrane interior on binding of domain T to artificial membranes. It is interesting to note that the residues photolabeled in the TH-9 region are similar to the residues identified in the spin labeling study (18). 5) Of late, detailed analysis of the protein fragments labeled by hydrophobic photoactivatable reagents has permitted identification of the structural motif of the labeled fragment depending on the side from which the reagent can bind and label the fragment in the native structure. Thus α-helix and β-sheets could be characterized by observing a periodicity of every third to fourth residue labeling in α-helices and alternate residues in β-sheets (21, 36, 37). When we analyze the photolabeling data reported here, one sees a certain level of periodicity in the labeling pattern which corresponds to the parent structural motif. Thus in CN-9 the β-sheet RB-7 is labeled at alternate residues 469, 471, 473, and 475, suggesting that DAF is facing one side of the β-strand. Similarly in the α-helix TH-9, every third residue, i.e. 365, 368, 371, and 374, is labeled, suggesting that one side of the α-helix is labeled. The periodicity is more pronounced in the middle of the motif than at the ends. A low pH-induced labeling of hydrophobic residues and periodicity of labeling, which strongly favor the retention of secondary structure, suggest that part of the DT molecule (domains T and possibly R) corresponds to the molten globule state. A low pH-induced folding of native proteins to the molten globule state where the secondary structure is retained but the tertiary structure is disrupted leading to exposure of hydrophobic sites has been detected in number of proteins (38, 39). More recently independent studies in the case of DT have also suggested the formation of molten globule state at low pH (40, 41). That a molten globule state may be involved in promoting insertion of soluble proteins in membranes has also been proposed earlier (42, 43).

Finally we will address the high labeling of the Pro-345 residues in TH-8. This residue belongs to the hydrophobic TH-8 and TH-9 region, which form a helical hairpin in the core of domain T and may be critical to membrane translocation. High labeling of Pro-345 indicates maximal hydrophobic exposure in this region. The high Pro-345 labeling leads us to speculate that this residue at low pH may be involved in a pH-induced isomerization of the Xaa-Pro imide bond from trans to cis, the Ile-Pro bond in DT being in trans geometry at neutral pH as reported in the crystal structure (4). It is interesting to note that acid-induced isomerization of the Xaa-Pro bond has been reported in the literature (44–46). The data given in Fig. 6 clearly indicate increased labeling of Pro-345 when the pH is lowered, with a sharp rise below pH 5.6. These results point to the important role of Pro-345 in a pH-induced conformational change in domain T of DT. The strongest evidence in favor of this result comes from the mutation of Pro-345 in DT to Glu or Gly, which leads to a 99% reduction in cytotoxicity without affecting the catalytic or receptor binding activity (47). Our preliminary molecular modeling studies using coordinates of DT show that the Ile-Pro isomerization from trans to cis form leads to hydrophobic TH-9 helix moving from the protein interior to the aqueous environment, which could initiate the initial binding of DT to membranes. However, at this stage the Ile-Pro trans-cis isomerization proposal is based on only circumstantial evidence and will require more definite evidence for its confirmation.

In conclusion, we have been able to identify the hydrophobic sites in DT which are exposed upon lowering of pH. The major labeled sites correspond to the putative membrane-inserting helices TH-8 and TH-9 and notably the Pro-345 residue, which has a crucial role to play in pH-dependent conformational change. Finally, the labeling pattern indicates that the parent structural motifs in DT are retained, suggesting that DT forms the classical molten globule intermediate at low pH. The DAF labeling thus provides an independent method to detect and identify hydrophobic residues exposed in molten globule states in proteins, information that at present can only be obtained using the NMR H-D exchange method. However, the NMR method can only be applied to small proteins like apomyoglobin (48) and not to large proteins (39, 49), which is a serious limitation. This limitation does not apply to the method reported here using DAF. To the best of our knowledge this is the first study on characterizing hydrophobic sites exposed in a soluble protein by peptide sequencing of the photolabeled protein.

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