Pharmaceutical Research, Vol. 22, No. 7, July 2005 (© 2005)
DOI: 10.1007/s11095-005-5643-3

Research Paper

Optimization and Applications of CDAP Labeling for the Assignment of Cysteines

Gary D. Pipes,[1] Andrew A. Kosky,[1] Jeffrey Abel,[1] Yu Zhang,[1] Michael J. Treuheit,[1] and Gerd R. Kleemann[1,2]

Received March 15, 2005; accepted April 29, 2005

Purpose. The aim of the study is to provide a methodology for assigning unpaired cysteine residues in proteins formulated in a variety of different conditions to identify structural heterogeneity as a potential cause for protein degradation.
Methods. 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) was employed for cyanylating free cysteines in proteins and peptides. Subsequent basic cleavage of the peptide bond at the N-terminal side of the cyanylated cysteines provided direct information about their location.
Results. CDAP was successfully employed to a wide variety of labeling conditions. CDAP was reactive between pH 2.0 and 8.0 with a maximum labeling efficiency at pH 5.0. Its reactivity was not affected by excipients, salt or denaturant. Storing CDAP in an organic solvent increased its intrinsic stability. It was demonstrated that CDAP can be employed as a thiol-directed probe to investigate structural heterogeneity of proteins by examining the accessibility of unpaired cysteine residues.
Conclusion. CDAP is a unique cysteine-labeling reagent because it is reactive under acidic conditions. This provides an advantage over other sulfhydryl labeling reagents as it avoids potential thiol-disulfide exchange. Optimization of the cyanylation reaction allowed the utilization of CDAP as a thiol-directed probe to investigate accessibility of sulfhydryl groups in proteins under various formulation conditions to monitor structural heterogeneity.

KEY WORDS: chemical cleavage; 1-cyano-4-dimethylaminopyridinium tetrafluoroborate; cyanylation; sulfhydryl assignment; thiol-disulfide exchange.

INTRODUCTION

Maintaining structural stability of proteins is one of the most challenging tasks in the development of liquid protein pharmaceuticals. The most common physical instability is protein aggregation, which can be introduced or affected or both by conformational heterogeneity and chemical transformations. Careful examination of these instability-influencing factors may help to prevent or mitigate certain stability problems. In addition, selection of proper and adequate analytical methods for efficient and accurate monitoring of protein instabilities may ensure successful development of quality protein products.

Disulfide bonds are often critical in controlling both protein activity and stability (1). Free cysteine residues in proteins can easily oxidize to form disulfide bond linkages or instigate thiol-disulfide exchange potentially causing protein aggregation or polymerization (2). However, if these cysteinyl residues are buried within the tertiary structure of proteins, they are much less reactive and less concerning.

Traditionally, because of the reactivity of free sulfhydryl groups, alkylating reagents such as iodoacetic acid or 4-vinyl pyridine have been extensively used to protect free cysteine residues to prevent oxidation or thiol-disulfide exchange (3,4). However, these reagents require basic pH (~pH 8.0) for optimal reactivity at which thiol-disulfide exchange apparently proceeds on the same time scale as alkylation (5). This does lead to rearrangements of folding intermediates during trapping with iodoacetate as observed for both Bovine Pancreatic Trypsin Inhibitor (BPTI) (6) and ribonuclease A (7). Therefore, a reagent that would alkylate free sulfhydryl groups under acidic conditions would be desirable to obtain valuable data for assigning free cysteine residues by maintaining the native disulfide linkages. An alternative reaction to protect sulfhydryl groups is cyanylation, which has been known and used for decades (8–10). A number of side reactions have been described in the use of cyanylation for labeling cysteine residues (10–13). Despite these, interest in its use has continued. New cyanylation reagents that show promise such as 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) have been developed (14), and the conditions for successful use of this reagent have been ex-

[1] Department of Pharmaceutics, MS 2-1-A, Amgen Inc., One Amgen Center Drive, Thousand Oaks, California 91320-1799, USA.
[2] To whom correspondence should be addressed. (e-mail: gerdk@amgen.com)

ABBREVIATIONS: CDAP, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate; DAP, 4-dimethylaminopyridinium tetrafluoroborate; GdnHCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; OPG, osteoprotegerin; rmethG-CSF, recombinant methionyl human granulocyte-colony stimulating factor; TOF, time of flight; UV, ultraviolet.

plored (14–16). As illustrated in Fig. 1, the reagent, CDAP, selectively cyanylates sulfhydryl groups of unpaired, reduced cysteine residues under acidic conditions (8,15,17) resulting in a shift of +25 Da in molecular weight (18). Subsequent basic cleavage of the protein chain (8,18,19) and reversed-phase high-performance liquid chromatography (HPLC) in conjunction with mass mapping by in-line mass spectrometric analysis provides a simple methodology to identify the location of unpaired cysteine residues as well as disulfide-linked cysteines. Because this labeling reaction can be performed in the absence and in the presence of strong denaturants [guanidine hydrochloride (GdnHCl), urea, SDS, etc.], it can offer direct insights into the surface accessibility of unpaired cysteine residues. Furthermore, protecting cysteines under acidic pH diminishes thiol-disulfide exchange by reducing the concentration of reactive thiolate anion (6).

For formulation work, the pH of the formulated protein solution and the excipients present should not interfere or inhibit the labeling reaction. Prior work utilizing CDAP has not adequately addressed the impact of pH and excipients on CDAP labeling reactivity. We conducted studies with a model peptide to investigate CDAP labeling dependency on these variables to add to the knowledge of its stability and factors that influence labeling reactivity. The objective of this study was to learn how to control the labeling variables and use this methodology to probe the structure of various proteins.

MATERIAL AND METHODS

Chemicals

All chemicals were ACS grade or better. 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) was purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. GdnHCl was a product of Boehringer-Mannheim Biochemicals (Indianapolis, IN). $CuCl_2$ and NaOH were purchased from Sigma-Aldrich. The standards (trypsinogen, insulin, and sinapinic acid) for the mass spectrometer calibration were purchased from Sigma-Aldrich. HPLC grade solvents were obtained from Burdick and Jackson (Muskegon, MI). Trifluoroacetic acid (TFA) was obtained from Baker (Rockford, IL).

Polypeptides and Proteins

The Fc–osteoprotegerin (OPG) fusion protein containing the Fc domain of an IgG1 monoclonal antibody, as well as the cytokine osteoprotegerin (OPG), and recombinant methionyl human granulocyte-colony stimulating factor (rmethG-CSF), were prepared by Amgen, Inc., Thousand Oaks, CA. The CYS-2 peptide (acetyl-FLLKCLE-amide; ε_{280} = 120 M^{-1} cm^{-1}) was synthesized using standard solid phase chemistries at Amgen, Inc., Boulder, CO.

Instrumentation

The HPLC systems used consisted of a Hewlett Packard 1090 HPLC system equipped with a diode array detector and controlled by ChemStation software (Wilmington, DE), an Agilent Technologies 1100 series HPLC system (Wilmington, DE) equipped with a diode array detector and controlled by ChemStation software, and a Michrom BioResources Magic 2002 HPLC system (Auburn, CA) equipped with a dual wavelength detector. For mass spectrometric analysis, the HPLC systems were coupled either to a Perkin-Elmer/Sciex Atmospheric pressure ionization (API) 100 mass spectrometer (Thornhill, Ontario, Canada) or a Thermo Finnigan LCQ Deca ion trap (Waltham, MA) mass spectrometer. The Kratos Kompact MALDI IV TOF mass spectrometer (Kratos Analytical Shimadzu Corp., Columbia, MD) was equipped with a curved field reflectron and pulsed extraction.

HPLC Separation

The separation of cyanylated CYS-2 peptide was achieved by reversed-phase chromatography using an Agilent Technologies 1100 series or a Michrom BioResources Magic 2002 HPLC system. All HPLC analyses were monitored by ultraviolet (UV) detection at 215 nm. Linear gradient elution was performed on a Zorbax SB-AQ column (Agilent Technologies; 5-μm particle size, 300-Å pore size, 2.1 × 150 mm) using a mobile phase comprising 0.1% TFA aqueous solution (mobile phase A) and 90% CH_3CN containing 0.1% TFA (mobile phase B) with a flow rate of 0.2 mL/min and a column temperature of 45°C. Fc-OPG main peak, CDAP-labeled post peak, and Fc-OPG cleavage products were separated by reversed-phase HPLC using an Agilent Technologies 1100 series HPLC system employing a linear gradient on a Zorbax 300SB C8 column (Agilent Technologies; 5-μm particle size, 300-Å pore size, 4.6 × 250 mm) with a flow rate of 1 mL/min and a column temperature of 60°C. The mobile phase composition was the same as for the CYS-2 peptide. UV detection was performed at 215 nm. For peak collection, Fc-OPG main peak and CDAP-labeled post peak were separated by semipreparative reversed-phase HPLC using a HP 1090 HPLC employing a linear gradient on a Zorbax 300SB C8 column (Agilent Technologies; 5-μm particle size, 300-Å pore size, 9.4 × 250 mm) with a flow rate of 4 mL/min and a column temperature of 60°C. The mobile phase composition was the same as for the CYS-2 peptide. Eluted peptides and proteins were analyzed directly by in-line electrospray ionization (ESI) mass spectrometry.

Mass Spectrometry

Matrix-assisted laser desorption/ionization (MALDI) mass spectra of CDAP and CDAP degradation products were obtained on a Kratos Kompact MALDI IV TOF mass spectrometer (Kratos Analytical Shimadzu Corp.). The accelerating voltage was set to 10.0 kV, and the laser power was optimized between 60 and 80. Samples of CDAP (0.2–0.5 μl) were applied directly to the stainless-steel sample slide and air-dried. No matrix was added. Spectra were generated in positive ion mode. Time-to-mass conversion was achieved by external calibration using standards of Na [Molecular weight (MW) = 22.99] and sinapinic acid (SA; MW = 224.21). All ESI mass measurements of the CYS-2 peptide were performed on a Thermo Finnigan LCQ Deca ion trap. The source voltage was set at 5.0 kV, the capillary at 7.0 V, the N_2 pressure at 68 psi, and the heated capillary at 250°C. ESI mass spectra of Fc-OPG were obtained on a Perkin-Elmer/Sciex API 100. The API source voltage was set at 3.5 kV, the cone voltage at

55.0 V, and the skimmer at 2.5 V. The N_2 pressure was 65 psi, and the capillary temperature was at ambient temperature.

Cyanylation of Sulfhydryl Groups

Cyanylation of the CYS-2 peptide was carried out using CDAP solutions (12 mg/mL) in 0.1 N HCl or neat acetonitrile. The CDAP solutions were added in 1:2 or 10:1 molar ratios of CDAP over sulfhydryl content as noted. Cyanylation of the sulfhydryl groups was accomplished by incubation of the mixture at 29°C for 15 min, except as noted. For cyanylation of Fc-OPG, CDAP was prepared in 0.5 M HCl at a concentration of 11 mg/mL, and 100 μL was added to 10 mg/mL of Fc-OPG (12:1 molar ratio of CDAP to Fc-OPG sulfhydryls) formulated in 10 mM sodium acetate pH 5.0 containing 5.0% sorbitol. The reaction mixture was incubated at 29°C for 15 min. Cyanylation of rmethG-CSF at pH 7.0 (100 mM Tris–HCl, pH 7.0) and pH 4.0 (100 mM sodium acetate, pH 4.0) was performed with dilutions from a 12.0-mg/mL stock solution of CDAP in neat acetonitrile. The CDAP-to-cysteine ratio was adjusted to 10:1. The labeling reactions were carried out at 29°C for 15 min.

Alkaline Cleavage of Cyanylated Peptides and Proteins

Cleavage of cyanylated CYS-2 peptide was performed at 37°C using 1.5 M NH_4OH. Typically, 5–20 μL of base was added to adjust the pH to 10.0–10.5. The peptide concentration was maintained at 0.5–1.0 mg/mL. The cleavage reaction was quenched by acidifying (pH 2–3) the mixture with TFA prior to reversed-phase HPLC separation and mass spectrometric analysis. Cyanylated Fc-OPG was purified by semipreparative reversed-phase HPLC, collected, and concentrated under vacuum by reducing the volume by approximately two thirds. The concentrated fractions were dissolved with 1.5 M NH_4OH containing 8.0 M GdnHCl to a final concentration of 4.0 M GdnHCl and 1.0 M NH_4OH and incubated at 37°C for 60 min. Cyanylated rmethG-CSF was processed by collecting the desired reversed-phase HPLC peak, which was then dried under vacuum. The cyanylated rmethG-CSF was resuspended using 1.5 M NH_4OH containing 4.0 M GdnHCl and incubated at 37°C for 2 h. The reaction was acidified with 10% formic acid to achieve a pH of 3.0. Samples were stored at −20°C or immediately analyzed by reversed-phase HPLC/mass spectrometry (MS).

Copper-Aided Disulfide Formation

Fc-OPG was diluted to 1 mg/mL using 10 mM sodium acetate, pH 5.0, 5.0% sorbitol buffer. $CuCl_2$ was added to a final concentration of 50 mM. The mixture was allowed to incubate at 29°C for 60 min and was then dialyzed against 10 mM sodium acetate, pH 5.0, 5.0% sorbitol buffer overnight. The dialysis buffer was changed once after 6 h.

RESULTS AND DISCUSSION

Intrinsic Stability of CDAP

The cyanylation of sulfhydryl groups had first been accomplished by Jacobson *et al.* (8) who utilized 2-nitro-5-thiocyanobenzoic acid (NTCB) under alkaline conditions (pH 8.0–9.0). NTCB had been reported to be specific to sulfhydryl groups, although side reactions such as the formation of mixed disulfide bonds between NTCB and the protein thiol groups had been reported (10–13). Furthermore, cyanylation by NTCB is slower than alkylation by iodoacetate (20), and because the reaction by NTCB must be performed under alkaline conditions, thiol-disulfide exchange and disulfide scrambling potentially have a competitive advantage over the cyanylation reaction.

A useful alternative to NTCB proved to be CDAP, which had been reported to be a selective and reactive cyanylation reagent for free sulfhydryl groups under acidic conditions, pH 3.0–5.0 (14,15). Therefore, CDAP would be the preferable reagent because of minimization of thiol-disulfide exchange under acidic labeling conditions. An optimal set of conditions for the selective cyanylation of sulfhydryl groups were previously determined to be a 10-fold molar excess of CDAP (over sulfhydryl groups) in pH 3.0 buffer with an incubation time of 10–15 min at ambient temperature (18). Control of pH is very important for successful cyanylation by CDAP, as lower pH (<pH 2.0) would slow down the reaction significantly, whereas higher pH (>pH 4.5) would catalyze hydrolysis of the CDAP reagent (20). Therefore, even under the optimal reaction conditions of pH 3.0, an excess of CDAP over sulfhydryl groups is necessary to compete with CDAP degradation through the formation of 4-dimethylaminopyridinium tetrafluoroborate (DAP). As most reactions involving biological materials are performed in aqueous solvents, the breakdown of CDAP cannot be avoided. For this reason, CDAP solutions for cyanylation reactions have to be prepared fresh, which makes the preparation of stock solutions for sequences of reactions over long periods of time unfeasible.

However, as demonstrated in this study, the rate of CDAP degradation can be slowed significantly if stored in organic solvents (Fig. 2). The MALDI Time-of-Flight (TOF) mass spectra shown in Fig. 2A represent CDAP stored in either aqueous (0.1 N HCl, pH 3.0) or organic (neat acetonitrile) solvents for 24 h at 29°C. CDAP degradation was determined by measuring the decay of CDAP (m/z = 149.1) to 4-dimethylaminopyridinium (DAP; m/z = 124.1) through integration of the corresponding MS signals from the MALDI-TOF mass measurements. The half-life ($t_{1/2}$) for CDAP when stored in 0.1 N HCl, pH 3.0, was determined to be 2.7 h (Fig. 2B). In contrast, CDAP stored in acetonitrile for the same period of time at 29°C remained stable and did not show any significant degradation. In agreement with CDAP degradation was the labeling efficiency of a single cysteine-containing peptide (CYS-2, acetyl-FLLKCLE-amide) by CDAP relative to the time CDAP was stored in either 0.1 N HCl, pH 3.0, or neat acetonitrile (Fig. 2B). The labeling efficiency as a measure for CDAP degradation was determined by the increase and decrease of the respective chromatographic peak areas of cyanylated and noncyanylated CYS-2 peptide (Fig. 3). In all experiments, the molar CDAP-to-sulfhydryl ratio was set to 1:2 to limit the maximum amount of label to 50%, enabling the detection of small changes in labeling efficiency. The result was that the labeling efficiency decreased with a half-life ($t_{1/2}$) of 2.3 h when the CYS-2 peptide was cyanylated by CDAP that was

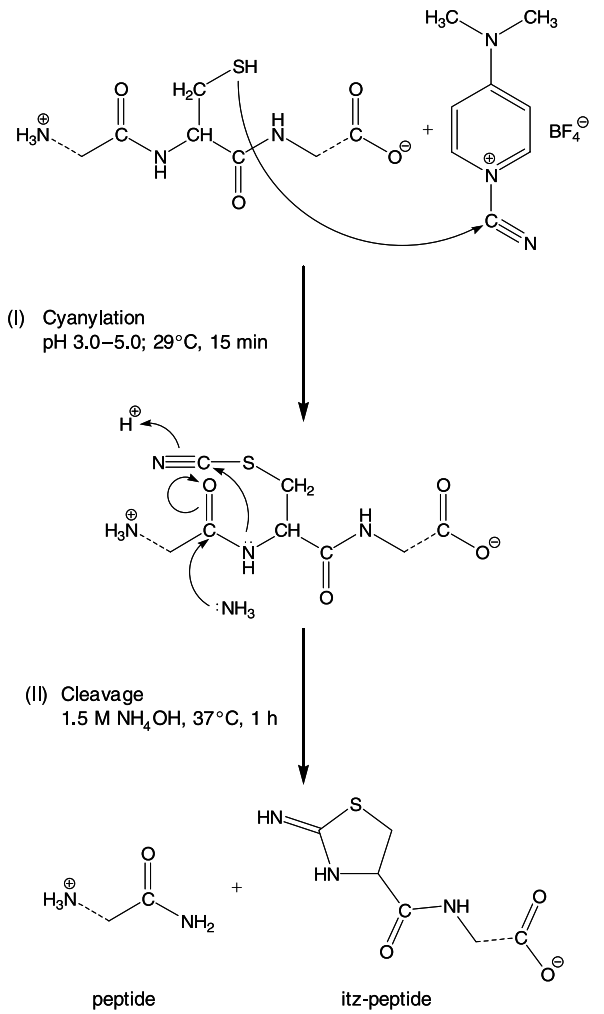

Fig. 1. Reaction mechanism between cysteine residue and 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP); (I) cyanylation of sulfhydryl group by CDAP and (II) specific peptide bond cleavage catalyzed by ammonia; itz, iminothiazolidine derivative. The shown schematic is based on the published mechanism by Wu and Watson (18).

incubated in aqueous solvent. This result is in close agreement with the degradation kinetics of CDAP itself ($t_{1/2}$ = 2.7 h; Fig. 2B) and is therefore a measure of reactive CDAP in solution. CYS-2 peptide cyanylated by CDAP stored in acetonitrile did not show any decrease in labeling efficiency, which is in good agreement with the chemical stability of CDAP (Fig. 2A) under these conditions.

The described work indicated that if CDAP is prepared in organic solvents like acetonitrile, it is possible to store the CDAP stock solutions for long periods of time to perform repetitive types of experiments without losing reactive CDAP to hydrolysis.

CDAP Labeling Efficiency Under Different Reaction Conditions

For protein formulation development, the pH of the formulated protein or peptide solution and the excipients present should not interfere with or inhibit the labeling reaction by CDAP. Because there is a lack of sufficient data available on the impact of pH and excipients on CDAP labeling efficiency, studies were conducted to investigate the CDAP labeling dependency of those variables.

We were interested in the applicability of CDAP labeling of proteins in a wide range of formulation pHs. The effect of pH on CDAP labeling efficiency was investigated in greater detail using the CYS-2 peptide. The CYS-2 peptide was formulated in sodium acetate, citrate, and phosphate buffers with a pH ranging from pH 2.0 to 8.0 and reacted under CDAP-limiting conditions (CDAP/CYS-2 peptide ratio of 1:2). Under these conditions, the labeling efficiency is expected to be 100.0%, when 50.0% of the peptide population becomes labeled. The reaction products were separated by reversed-phase HPLC and identified and quantified by mass spectrometric analysis using an ion trap mass spectrometer. As illustrated in Fig. 4, the maximum CDAP labeling efficiency was found to be at pH 5.0, at which approximately 50.0% of the CYS-2 peptide became cyanylated corresponding to 100% labeling efficiency under the specified limiting conditions. At pH < 5.0, only 25.0% of the CYS-2 peptide was cyanylated with the labeling efficiency dropping to 50.0%. Because the thiolate anion is the reactive species in thiol-disulfide exchange, the cyanylation activity is quenched at low pH because of the protonation of the cysteine's thiolate anion effectively reducing the degree of cyanylation. These results deviate from the previously published pH optimum at pH 3.0 (14,18). However, both groups reported that CDAP effectively labels sulfhydryl groups in the pH range between pH 3.0 and 5.0, consistent with our findings. Labeling with CDAP at pH > 5.0 is competing with two other reactions: the increasing degradation rate of CDAP itself (Fig. 2) and with the formation of a disulfide-linked CYS-2 dimer. This demonstrated the increasing risk of free thiol-induced disulfide exchange in proteins with increasing pH. Therefore, with increasing CDAP degradation and dimer formation, the cyanylation of the CYS-2 peptide decreased to about 15.0% at pH 8.0. However, while less reactive at higher pH using the CYS-2 peptide, CDAP labeling can still yield useful results for analyzing unpaired cysteine residues in proteins (refer to the next section).

In a second study, the CYS-2 peptide was used to demonstrate the applicability of CDAP as a labeling reagent for proteins that are formulated in the presence of a variety of buffers, excipients, and a common denaturant. CYS-2 was formulated in sodium acetate, citrate, and histidine buffers with a concentration of 10 mM and a pH of 5.0. Sodium acetate (10 mM) at pH 5.0 was also used to independently examine the following excipients and denaturant: 0.0–1.0 M sodium chloride, 5.0% sorbitol, 9.0% sucrose, 0.1% polysorbate 20, 5.0% arginine, 5.0% glycine, 2.0% mannitol, and 0.0–5.0 M guanidine hydrochloride. The CDAP labeling reaction was performed under CDAP-limiting conditions (CDAP/CYS-2 peptide ratio of 1:2) at 29°C for 15 min, and the reaction products were quantified by reversed-phase HPLC/MS (Fig. 3). As summarized in Table I, the percentage of labeled CYS-2 peptide in all formulations tested ranged between 40 and 52% with an average standard deviation of about 3%. Taking into account that the labeling was performed under CDAP-limiting conditions, the expected maximum amount of labeled CYS-2 peptide would be 50% with

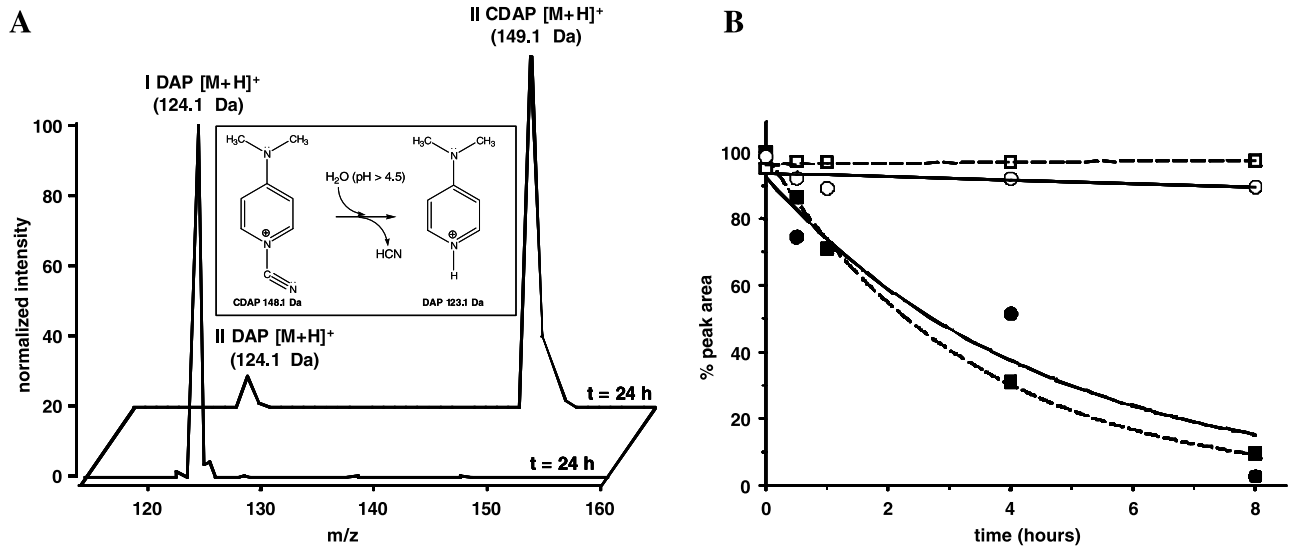

Fig. 2. Comparison of CDAP degradation rates in aqueous *vs.* organic solvent. Panel A shows the MALDI-TOF spectra of CDAP (m/z = 149.1) and its degradation product, DAP (m/z = 124.1), after storage at 29°C for 24 h. The breakdown of CDAP in aqueous (I, 0.1 N HCl, pH 3.0) and organic (II, neat acetonitrile) solvents are compared. Panel B compares the decay of CDAP in aqueous (solid circles; 0.1 N HCl pH 3.0) and organic solvents (open circles; neat acetonitrile) *vs.* the labeling efficiency of the CYS-2 peptide by CDAP which has been stored for different periods of time in aqueous (solid squares; 0.1 N HCl, pH 3.0) and organic solvent (open squares; neat acetonitrile) at 29°C. The insert illustrates the CDAP degradation mechanism at pH > 4.5.

the labeling efficiency ranging from 80 to 103%. These data suggest that CDAP can be applied as a labeling reagent in the presence of a wide variety of excipients, surfactants, and denaturants and across pH values ranging from pH 3.0 to 8.0.

CDAP Labeling as a Thiol-Directed Probe for Examining Structural Heterogeneity

Disulfide linkages in proteins often confer chemical and physical stability (21,22) and have been proven to be important in the elucidation of protein-folding pathways (23–26). The loss of a single disulfide bond can potentially result in a secondary or tertiary structural change that can lead to chemical or physical instability (27–29).

Proteins, which have both disulfide bonds and free thiol groups, may aggregate via thiol-disulfide exchange. The rate of thiol-disulfide exchange depends on the extent of ionization of the nucleophilic thiol and, therefore, generally increases as the reaction pH increases (30).

We evaluated the possibility of using CDAP as a conformationally sensitive thiol-directed probe to test for the presence and accessibility of free, unpaired cysteine residues

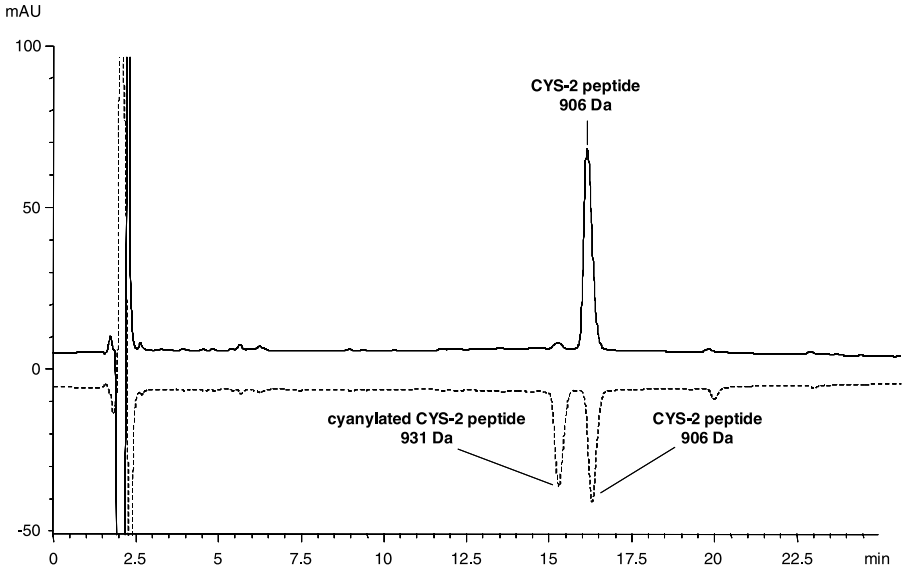

Fig. 3. Reversed-phase HPLC chromatograms of the CYS-2 peptide (acetyl-FLLKCLE-amide) before (solid line) and after partial cyanylation (dashed line) in 100 mM sodium acetate, pH 5.0, for 15 min at 29°C. The shift of +25 Da in molecular weight is the result of a single cyanylated cysteine residue.

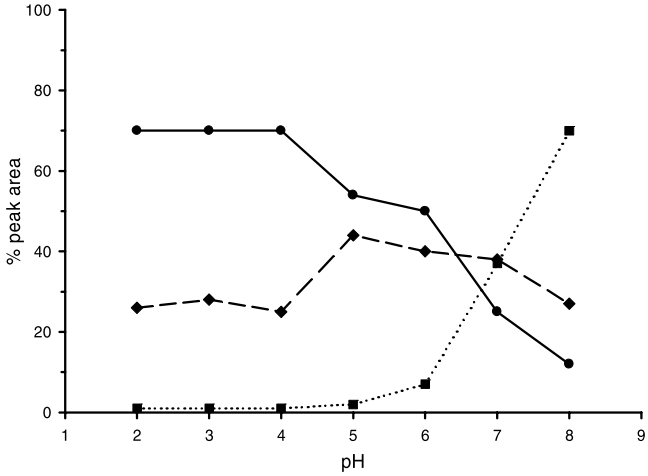

Fig. 4. The labeling efficiency of CDAP was greatest at pH 5.0. The CDAP labeling efficiency, as a function of pH, was determined by integrating the respective peak area of the cyanylated and noncyanylated CYS-2 peptide as analyzed by reversed-phase HPLC (Fig. 3). The solid circles (●) represent the decrease of nonlabeled CYS-2 peptide. The solid diamonds (◆) represent the increase of labeled CYS-2 peptide. The solid squares (■) represent the increasing formation of dimeric CYS-2 peptide. In all cases, the cyanylation reaction was performed under CDAP-limiting conditions (CDAP/CYS-2 peptide ratio 1:2).

in the structural context of a native protein fold. This approach has been successful when using spectroscopic probes (31) like fluorescein 5′-maleimide, 4-dimethylaminophenyl-azo-phenyl-4′-maleimide, and 4-dimethylaminophenyl-azo-phenyl-4′-iodoacetamide. The advantage of employing CDAP for the assignment of unpaired cysteine residues in comparison to the reagents mentioned above is that CDAP is active at acidic pH and therefore reduces the probability of competing thiol-disulfide exchange reactions. Furthermore, CDAP not only places a label on free sulfhydryl groups resulting in a shift of +25 Da in molecular weight, but subsequent base cleavage of the protein chain identifies the location and quantity of free sulfhydryl groups (Fig. 1). We have employed CDAP to examine conformational heterogeneity in proteins by probing the thiol accessibility of free sulfhydryl groups under native or nondenaturing formulation conditions.

In the first study, Fc-OPG was used as a model protein to characterize the state of the cysteine side chains under native conditions. Fc-OPG is a fusion protein containing the Fc domain of an IgG1 monoclonal antibody and the cytokine osteoprotegerin (OPG). Fc-OPG is a homodimeric, high molecular weight protein (91 kDa) containing 24 cysteines in each monomer primary sequence. All cysteine residues in the dimer are supposed to be covalently linked through 24 disulfide bonds, which are essential for the integrity of the tertiary and quaternary structure of the protein. However, characterization by reversed-phase HPLC revealed an isoform of Fc-OPG (Fig. 5). Mass spectrometric analysis of the main peak and the later eluting post peak, representing 12.0% of the total Fc-OPG, showed that they differ by only 2 Da, 90,722 and 90,724 Da, respectively (Fig. 5A). When Fc-OPG was reacted with aqueous $CuCl_2$, the post peak isoform eluted with the same retention time and molecular weight as the reversed-phase HPLC main peak (Fig. 5B). This result led to the speculation that the later eluting isoform probably contained free sulfhydryl groups that were converted to cysteine with the aid of copper as an oxidizing reagent. To support this hypothesis, Fc-OPG was treated with CDAP at pH 5.0 to cyanylate any free sulfhydryl groups. Cyanylation of Fc-OPG gave the same reversed-phase HPLC profile (Fig. 5C) as for Fc-OPG not reacted with CDAP (Fig. 5A). However, analysis by reversed-phase HPLC/MS revealed that the post peak of cyanylated Fc-OPG was heavier by +50 Da (m/z = 90,774) than the post peak (m/z = 90,724) for noncyanylated Fc-OPG. This result suggested that two cysteine sulfhydryl groups became cyanylated to give a mass increase of +50 Da resulting from the addition of two 26-Da cyanide groups and the loss of two protons from the cysteine side chains. The cyanylated isoform was no longer responsive to the addition of $CuCl_2$ (Fig. 5C) because cyanylated cysteine residues are not competent for disulfide formation. To identify the location of the cyanylated cysteine residues, the two peaks were collected, subjected to specific chemical cleavage on the N-terminal side of the cyanylated cysteines in aqueous ammonia, and reanalyzed by reversed-phase HPLC/MS (Fig. 6). Cleavage occurred at the N-terminal peptide bond of a cyanylated cysteinyl residue to form an amino-terminal peptide and a 2-iminothiazolidine-4-carboxyl peptide (itz). Hence, the resulting cleavage pattern observed for the cyanylated post peak differed from that of the main peak. The post peak cleavage reaction produced three distinct peaks, whereas the main peak reaction resulted in only one

Table I. Excipient-Dependent 1-Cyano-4-Dimethylaminopyridinium Tetrafluoroborate (CDAP) Labeling Efficiency Under CDAP-Limiting Conditions (CDAP/CYS-2 peptide ratio of 1:2)

Excipient	Percentage of labeled cysteine	Percentage of labeling efficiency[a]	Error (SD)[b]
Acetate[c]	44.7	89.4	2.4
Acetate[c], 5.0% sorbitol	44.3	88.6	2.5
Acetate[c], 9.0% sucrose	47.6	95.2	3.1
Acetate[c], 5.0% arginine	43.6	87.2	4.4
Acetate[c], 5.0% glycine	46.0	92.0	2.0
Acetate[c], 2.0% mannitol	47.0	94.0	1.1
Acetate[c], 0.1% polysorbate 20	43.5	87.0	1.1
Acetate[c], 0.5 M GdnHCl	47.2	94.3	3.2
Acetate[c], 1.0 M GdnHCl	51.7	103.3	0.4
Acetate[c], 2.0 M GdnHCl	44.5	88.9	3.3
Acetate[c], 3.0 M GdnHCl	40.7	81.3	0.2
Acetate[c], 4.0 M GdnHCl	41.1	82.2	0.5
Acetate[c], 5.0 M GdnHCl	43.2	86.3	3.5
Acetate[c], 0.1 M NaCl	40.0	80.0	5.4
Acetate[c], 0.4 M NaCl	41.3	82.6	4.3
Acetate[c], 1.0 M NaCl	41.8	83.5	4.5
Citrate[d]	44.3	88.6	1.0
Histidine[e]	48.9	97.8	2.4

[a] Labeling efficiency under CDAP-limiting conditions (CDAP/CYS-2 peptide ratio of 1:2) is 100%, when 50% of CYS-2 becomes labeled.
[b] The standard deviation (SD) was calculated with N = 3; where N equals the number of labeling reactions performed in each excipient formulation.
[c] Acetate: 10 mM sodium acetate, pH 5.0.
[d] Citrate: 10 mM citrate, pH 5.0.
[e] Histidine: 10 mM histidine, pH 5.0.

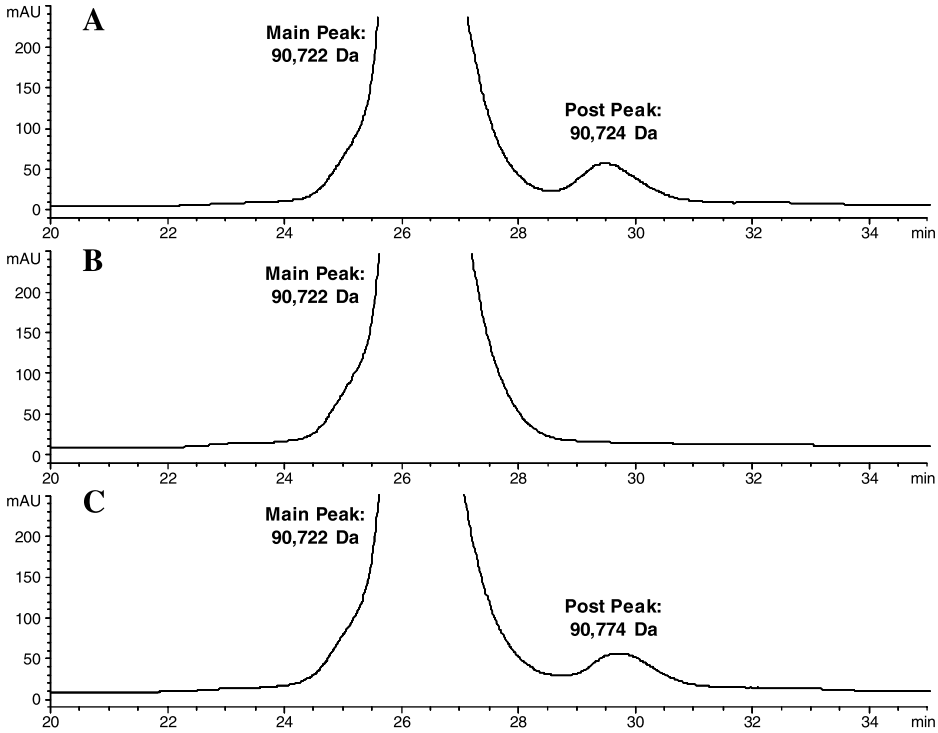

Fig. 5. The Fc-OPG post peak is reactive to $CuCl_2$ and CDAP. The various Fc-OPG forms were separated by reversed-phase HPLC and analyzed by UV and mass spectrometric detection. Panel A shows the reversed-phase HPLC chromatogram of unreacted Fc-OPG; panel B shows the reversed-phase HPLC chromatogram of Fc-OPG reacted with $CuCl_2$; panel C shows the reversed-phase HPLC chromatogram of Fc-OPG reacted with CDAP followed by $CuCl_2$. All peaks were labeled with the corresponding molecular weights as determined by HPLC/MS analysis.

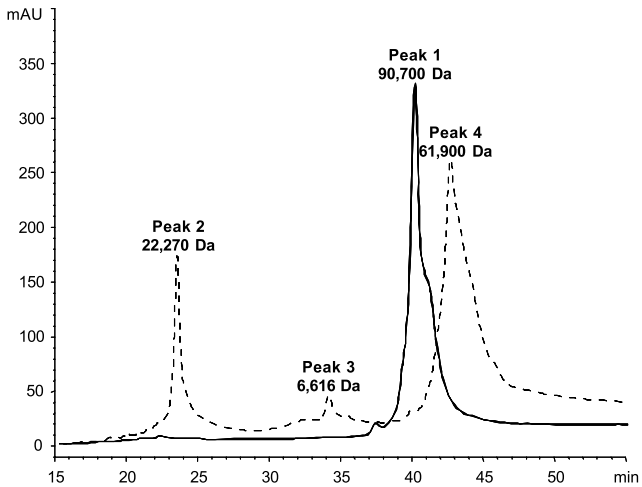

Fig. 6. The Fc-OPG post peak is reactive to basic pH cleavage by NH_4OH after CDAP labeling. CDAP-treated Fc-OPG was separated by reversed-phase HPLC (see Fig. 5). The peaks were collected and subjected to basic cleavage with NH_4OH and reanalyzed by reversed-phase HPLC/MS. The solid line UV trace represents purified main peak (Fig. 5; panel A) after cleavage with NH_4OH. The dashed line UV trace represents purified post peak (Fig. 5; panel A) after cleavage with NH_4OH. All chromatographic peaks are labeled with the corresponding molecular weights as determined by HPLC/MS analysis. For the theoretical molecular weights, refer to Table II.

peak (peak 1, Fig. 6). Mass analysis of the main peak gave the expected molecular weight for the intact Fc-OPG protein (90,700 Da) suggesting that none of the cysteine residues have reactive sulfhydryl groups. The mass analysis of the post peak cleavage products is summarized in Table II. Peak 2 with a molecular weight of 22,270 Da represents the C-terminal itz-fragment containing Cys-206 in the C_H3 region of the Fc domain (Fig. 7). Peak 3 has a molecular weight of 6,616 Da and was generated due to cleavage N-terminally of Cys-148, also located in the C_H3 region, resulting in the internal itz-fragment between Cys-148 and Cys-206 (Fig. 7).

Table II. Summary of the Cleavage Products of Fc-OPG Derived From Reversed-Phase HPLC Peaks in Fig. 6 and Measured by Mass Spectrometric Analysis

Protein	Peak	Measured peptide mass[a]	itz-Peptide adjusted mass[b]	Expected peptide mass[c]
Fc-OPG	1	90,700	n.a.	90,696
	2	22,270	22,245	22,247
	3	6,617	6,592	6,591
	4	61,900	none	61,894

[a] Mass determined by mass spectrometry (Da).
[b] Adjusted mass (Da) by subtracting 25 Da to account for cyanide (CN) in the itz-peptide.
[c] Theoretical value (Da) from known amino acid sequence.

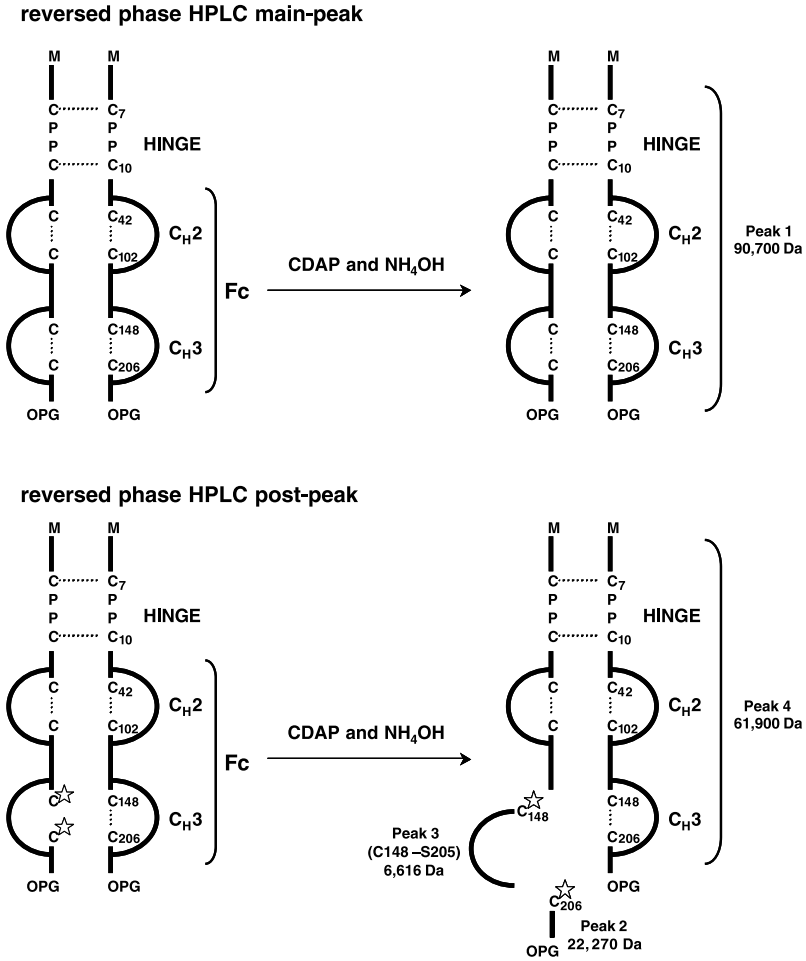

Fig. 7. Schematic illustration of the Fc-OPG main peak *vs.* post peak with and without the Cys-148/Cys-206 disulfide bond present. The cleavage products comprising the Cys-206 containing C-terminal itz-fragment (Peak 2; Fig. 6), the Cys-148 containing internal itz-fragment (Peak 3; Fig. 6), and the truncated remainder of Fc-OPG (Peak 4; Fig. 6) are also illustrated. The fragments are labeled with the corresponding molecular weights determined by HPLC/MS analysis. Cys-148 and Cys-206 marked with a star (☆) are not disulfide bound in the post peak but accessible to cyanylation by CDAP and reactive to basic cleavage by NH_4OH.

Peak 4 has a mass of 61,900 Da and represents the remainder of Fc-OPG with a truncated Fc domain (Fig. 7). These results revealed two free cysteine sulfhydryl groups located in the C_H3 domain in one of the two monomeric chains of the Fc-OPG fusion protein. In subsequent experiments, the reversed-phase HPLC post peak could be converted to main peak under alkaline-denaturing conditions (data not shown). Apparently, formation of cysteine in the post peak was rapid enough under alkaline conditions to mask the structural heterogeneity and to go undetected by other techniques (Ellman's reaction and peptide mapping). The site specificity and acid compatibility of CDAP as a thiol-directed probe made it possible to identify and quantify unpaired cysteine residues in the C_H3 region of the Fc-OPG fusion protein.

In our second study, we investigated the thiol accessibility of Cys-17 (nomenclature from the G-CSF sequence lacking the N-terminal methionine) in recombinant methionyl human granulocyte-colony stimulating factor (rmethG-CSF) (32). rmethG-CSF is comprised of 175 amino acids and has a molecular weight of 18,798.8 Da. Its amino acid sequence includes five cysteine residues (Cys-17, Cys-36, Cys-42, Cys-64, and Cys-74). In the native structure, Cys-36 and Cys-42, as well as Cys-64 and Cys-74, are paired in intramolecular disulfide bonds, whereas Cys-17 exists in its reduced form and is not involved in any disulfide bonding.

rmethG-CSF tends to aggregate under conditions greatly favoring the native state (e.g., physiological conditions: 37°C at neutral pH) *in vitro* (33). Previous studies propose that under these conditions, aggregation proceeds through a transition state species within the ensemble that is only slightly perturbed in structure relative to the most compact species (34). Studies investigating reengineered G-CSF mutants (35) show that enhancing the α-helix propensities of residues in the antiparallel 4-helix bundle of rmethG-CSF increases the overall thermodynamic stability of the protein. At the same time, with increasing thermodynamic stability, the rate of aggregation is decreasing and suppressed even further when Cys-17 is replaced by serine (36). A similar

effect on the rate of aggregation is observed when rmethG-CSF is formulated at pH 4.0. At acidic pH (between pH 3.3 and 4.5), rmethG-CSF is thermodynamically more stable because of an increase in helical content (37,38). Using 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) as a thiol-directed probe at pH 7.5, it was found that Cys-17 is partially solvent-exposed suggesting that it might play a critical role in the instability of the protein at neutral pH (36). However, the solvent exposure was not examined at pH 4.0 because of the inactivity of DTNB at acid pH. To this end, we used CDAP as an alternative thiol-directed probe to explore differences in the conformational environment of Cys-17 at pH 7.0 and 4.0.

rmethG-CSF was cyanylated at pH 7.0 and 4.0 under both denaturing (4.0 M GdnHCl) and native conditions using a 10-fold molar excess of CDAP over protein sulfhydryls and analyzed by reversed-phase HPLC/MS analysis. In neither case, whether rmethG-CSF was reacted with CDAP at pH 7.0 or 4.0, did the corresponding chromatographic profile change compared to the nonreacted rmethG-CSF control. However, deconvolution of the respective mass spectra revealed that after cyanylation by CDAP at pH 7.0 under denaturing as well as native conditions, the chromatographic main peak contained two different rmethG-CSF species with molecular weights of 18,798 and 18,823 Da, differing by +25 Da (data not shown). In contrast, at pH 4.0, rmethG-CSF became only cyanylated under denaturing conditions as confirmed by the increase in molecular weight (18,796 Da) by +24 Da (18,820 Da; data not shown). At pH 4.0, under native conditions, no cyanylated rmethG-CSF could be detected. The amount of cyanylated rmethG-CSF under native and denatured conditions at pH 7.0 was estimated from the deconvoluted mass spectrum peak areas to be 8.0 and 96.0%, respectively, indicating that under native conditions, Cys-17 is partially exposed at pH 7.0, whereas it seems to be completely protected against cyanylation at pH 4.0. The site of cyanylation was confirmed and identified by basic cleavage with aqueous ammonia and reversed-phase HPLC/MS analysis (data not shown).

Cys-17 under native conditions at pH 7.0 could be cyanylated, whereas at pH 4.0, no cyanylation was detectable. In agreement with previously published work indicating that rmethG-CSF is more compact and thermodynamically stable at or below pH 5.0 than at pH 7.0 (35–38), the results presented here also suggest that the conformation proximal to Cys-17 is more compact at pH 4.0 than at pH 7.0 because of a higher degree of protection toward cyanylation by CDAP.

CONCLUSION

With the objective to employ CDAP for the analysis of cysteines in proteins formulated in a variety of different conditions, we could demonstrate that CDAP is reactive in a wide pH range (2.0–8.0) with a maximum labeling efficiency at pH 5.0 (Fig. 4). This reactivity does not appear to be effected by certain excipients, salt or denaturant (Table I). We were able to increase the intrinsic stability of CDAP by storing it in organic solvents like acetonitrile (Fig. 2). Based on the optimized labeling properties and the compatibility to a wide range of labeling conditions, we successfully employed CDAP as a thiol-directed probe to identify pH-dependent structural differences in rmethG-CSF because of changes in the accessibility of the free thiol group of Cys-17. Cyanylation of Fc-OPG resulted in the characterization of an isoform where two unpaired cysteine residues were identified. In both rmethG-CSF and Fc-OPG, the site specificity and acid compatibility of CDAP, as well as the subsequent basic cleavage of the protein chains, allowed for probing the accessibility and the accurate identification of the unpaired cysteines.

ACKNOWLEDGMENT

The author likes to thank David Brems of Amgen for helpful discussions and guidance.

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