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Conformation-dependent cleavage of staphylococcal nuclease with a disulfide-linked iron chelate

(protein cleavage/free radicals/protein folding/iron–peroxide complex)

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ABSTRACT **We report the synthesis and evaluation of (EDTA–2-aminoethyl) 2-pyridyl disulfide. By using this easily prepared cysteine-specific hydrophilic reagent, an ethylenediaminetriacetic acid–Fe^{3+} complex (EDTA–Fe) was covalently attached to a single genetically engineered cysteine residue in staphylococcal nuclease. Upon addition of the iron reductant ascorbate, the nuclease–EDTA–Fe conjugate underwent a protein self-cleavage reaction mediated by reactive oxygen species. Sequence analysis of the products indicated that cleavage occurs close in tertiary structure to the EDTA–Fe attachment site. In the presence of denaturants, the cleavage pattern changes and the reaction is limited to residues proximal in sequence to the cysteine attachment site. These results indicate that intramolecular protein cleavage reactions mediated by EDTA–Fe can be used to evaluate changes in protein conformation. The reagent described should be a useful tool in the structural mapping of nonnative protein states populated at equilibrium, such as the molten globule, that are frequently refractory to conventional structure analysis.**

There is an increasing need for new probes to study the topology of protein nonnative states, such as the molten globule and other folding intermediates, that can be populated at equilibrium (1, 2). Modern NMR techniques are revolutionizing the analysis of small proteins in solution, but they can be used to characterize partially folded molecules only under favorable conditions (3). Antibodies, proteases, and chemical probes have been used in the study of protein folding (4–8), but they are not well suited for mapping partially folded structure.

A new class of chemical probes was devised largely for footprinting studies of DNA (9, 10). These reagents generate reactive oxygen species that label surrounding structural elements by oxidative degradation. Several metal chelates, bound covalently (11–13) or by affinity (14–16) to a protein, are effective reagents for localized cleavage of polypeptide chains by reactive oxygen species. When EDTA–Fe is attached covalently to a protein, cleavage occurs close to the attachment site in the three-dimensional structure, suggesting that protein backbone segments brought into proximity in a partially unfolded protein could be identified by using this approach. Diffusible hydroxyl radicals (17, 18) and reagent-bound metal–peroxide species (13) have been proposed to explain the reactions of EDTA–Fe. These highly reactive species attack the protein polypeptide backbone and side chains, leading to chemical modification and protein fragmentation (19). Potential applications of these cleavage reagents include the analysis of protein–protein and protein–nucleic acid interactions and the structural characterization of nonnative protein states such as the molten globule (1, 2).

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Here, we report the synthesis and evaluation of (EDTA–2-aminoethyl) 2-pyridyl disulfide (EPD; Fig. 1, compound **1**) an easily prepared cysteine-specific hydrophilic reagent useful for reversibly conjugating ethylenediaminetriacetic acid to any free thiol group in a macromolecule. This reagent is a versatile tool that promotes intramolecular and localized protein cleavage. Staphylococcal nuclease was genetically engineered to introduce a single cysteine at position 28, and this variant, K28C, was used to characterize EPD–Fe as a protein cleavage reagent. Experimentation with a protein of known three-dimensional structure (20, 21) allowed the accessibility and proximity of cleavage sites to be assessed. Sequence analysis of K28C–EDTA–Fe fragmentation products identified several cleavage sites located close in tertiary structure to the reagent attachment site. In the presence of sodium dodecyl sulfate (SDS) or guanidinium chloride, cleavage at sites remote in linear sequence was not observed and the reaction was limited to residues proximal in sequence to the Cys-28 attachment site.

MATERIALS AND METHODS

General Details. All chemicals were reagent grade. All synthetic reactions were carried out under an argon atmosphere. Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} precoated plates (0.25 mm, Merck). Flash chromatography (22) was performed using silica gel 60 (230–400 mesh, Merck). High-performance liquid chromatography (HPLC) was carried out with a computer-controlled system (Rabbit HP; Rainin, Woburn, MA) and UV detection. NMR spectra were recorded with a Bruker VM 250 instrument and are reported in parts per million (ppm) downfield from Me_4Si. Coupling constants are reported in hertz. IR spectra were recorded using a Nicolet 5-SX FT-IR spectrometer. Mass spectra were recorded using a VG ZAB-SE high-resolution spectrometer with a fast atom bombardment (FAB xenon) ion source. Thiol groups were determined using Ellman's reagent (23). Iron was determined as the 1,10-phenanthroline complex (24). Nuclease concentration was determined by absorbance measurement at 280 nm (25). Amino acid analysis was performed on a 7300 Beckman Autoanalyzer. Peptides were sequenced with an Applied Biosystems 470A sequencer. SDS/PAGE was performed according to Schägger and von Jagow (26). The separating gel [16.5% total monomer, 6% crosslinker containing 13.3% (wt/vol) glycerol] was overlaid by a stacking gel (4% total monomer, 3% crosslinker). Sample buffer was 4% SDS, 12% (wt/vol) glycerol/2% (vol/vol) 2-mercaptoethanol/0.01% bromophenol blue/50

Abbreviation: EPD, (EDTA–2-aminoethyl) 2-pyridyl disulfide.

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mM Tris·HCl, pH 6.8. Nondenaturing PAGE was performed with the Phast System (Pharmacia LKB). Western blot analysis of peptides electroblotted onto nitrocellulose was performed as described (27). Synthetic peptides matching the last 9 and the first 10 nuclease residues were crosslinked to bovine albumin and used for the production of rabbit polyclonal antibodies (Babco, Richmond, CA). Antibodies were affinity-purified and negative controls with peptide-neutralized antibodies were included in all Western blot experiments.

Cystamine-(EDTA triethyl ester)$_2$ (Compound 3). EDTA triethyl ester (2) (28) (0.83 g, 2.2 mmol) and cystamine (0.111 g, 0.73 mmol) were dissolved in CH_2Cl_2 (7.0 ml) and the solution was cooled in an ice bath. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.492 g, 2.6 mmol) and 4-dimethylaminopyridine (catalytic amount) were then added with constant stirring. The ice bath was removed; the solution was stirred for 14 hr at room temperature, filtered, extracted with saturated aqueous NaCl (2 × 10 ml), dried (Na_2SO_4), and evaporated under reduced pressure. The crude product was purified by flash chromatography (MeOH/CH_2Cl_2, 6:94, vol/vol) to give 0.28 g (44% yield) of **3**. ^{1}H NMR (250 MHz, C^2HCl_3): δ 1.19 (t, 18H, J = 7.1, OCH_2CH_3), 2.78–2.87 (m, 12H, $N'CH_2CH_2N$ + NCH_2CH_2S), 3.28 (s, 4H, $N'CH_2CONH$), 3.40 (s, 4H, $N'CH_2CO_2Et$), 3.51 (s, 8H, NCH_2CO_2Et), 3.56 (t, 4H, J = 5.0, NCH_2CH_2S), 4.13 (q, 12H, J = 7.1, OCH_2CH_3). IR (neat, cm^{-1}): 2983, 1742, 1666, 1189, 1027. MS: m/z 869.9 (MH^+ calculated for $C_{36}H_{64}N_6O_{14}S_2$ = 869.4, 3-nitrobenzyl alcohol/trifluoroacetic acid matrix). TLC: R_f = 0.5 (SiO_2, MeOH/CH_2Cl_2, 1:9, vol/vol).

***N*-[2-[Bis(carboxymethyl)amino]ethyl]-*N*-(2-oxo-2-{[2-(2-pyridinyldithio)ethyl]amino}ethyl)glycine (EPD; Compound 1.** To a solution of **3** (0.26 g, 0.3 mmol) in 10 ml of ethanol was added 25 ml of 0.5 M LiOH. The solution was stirred at room temperature for 2 hr, then acidified to pH 4.0 with concentrated HCl. The organic solvent was removed under reduced pressure, and the aqueous solution was freeze-dried. ^{1}H NMR (250 MHz, ^2H_2O): δ 2.73 (t, 4H, J = 6.0, NCH_2CH_2S), 2.95 (bt, 4H, NCH_2CH_2N), 3.18 (s, 4H, $N'CH_2CONH$), 3.27 (bt, 4H, NCH_2CH_2N), 3.32 (s, 4H, $N'CH_2CO_2H$), 3.43 (t, 4H, J = 6.0, $NHCH_2CH_2S$), 3.71 (ds, 8H, NCH_2CO_2H). IR (KBr, cm^{-1}) 3366 (b), 2525, 1630, 1435, 1415. The hydrolyzed product was dissolved in water (2 ml, deaerated), the pH was adjusted to 6.0 (0.5 M LiOH), and 2-mercaptoethanol (1.0 ml, 12 mmol) was added. After 16 hr at room temperature the pH was adjusted to 4.0 (1 M HCl), and the solution was extracted with ethyl acetate (3 × 10 ml). The aqueous phase was freeze-dried, dissolved in water (4 ml, deaerated), and added to 4 ml of 0.5 M 2,2′-dipyridyl disulfide (Aldrich) in ethanol. The pH was brought to 6.0 (0.5 M LiOH), and the reaction mixture was incubated at room temperature for 3 hr. The solution was rotary-evaporated to 4 ml, and precipitated 2,2′-dipyridyl disulfide was removed by filtration. 2-Thiopyridone and residual 2,2′-dipyridyl disulfide were removed by extraction with CH_2Cl_2 (5 × 100 ml), and the solution was freeze-dried. The amount of mixed disulfide **1** was determined by measuring the 2-thiopyridone release upon disulfide exchange with 1,4-dithiothreitol (29). The yield was 32% based on **3**. EPD was purified further, from associated LiCl, by HPLC (Vydac C_{18} column, 4.6 mm × 25 cm) with a linear elution gradient (0.05% trifluoroacetic acid/25% acetonitrile in 0.05% trifluoroacetic acid, 1 ml/min, 1%/min) and absorbance detection at 290 nm. As HPLC analysis showed that EPD was >95% of the UV-absorbing material, the crude lithium salts/EPD mixture was complexed with Fe^{3+} and used in the protein modification experiments. ^{1}H NMR (250 MHz, ^2H_2O): δ 2.97 (t, 2H, J = 6.2, NCH_2CH_2S), 3.14 (t, 2H, J = 5.0, NCH_2CH_2N), 3.36 (t, 2H, J = 5.0, NCH_2CH_2N), 3.48 (t, 2H, J = 6.0, NCH_2CH_2S), 3.58 (s, 4H, $N'CH_2CONH$ + $N'CH_2CO_2H$), 3.87 (s, 4H, NCH_2CO_2H), 7.66 (t, 1H, J = 6.8, Pyr H-5), 8.09 (d, 1H, J = 8.0, Pyr H-3), 8.26 (t, 1H, J = 7.9, Pyr H-4), 8.52 (d, 1H, J = 5.6, Pyr H-6). MS: m/z 461.3 (MH^+ calculated for $C_{17}H_{24}N_4O_7S_2$ = 461.1, 3-nitrobenzyl alcohol/trifluoroacetic acid matrix). IR (KBr, cm^{-1}) 3395, 1633, 1445, 1421. EPD-Fe^{3+}, **4**, was prepared by dissolving **1** (21 μmol) in 0.13 M NaOH (410 μl) and adding 100 mM $FeCl_3$ (400 μl). After 20 min at room temperature the complex was purified from free iron by ion-exchange chromatography (Dowex 50 X8, Na^+ form, 5 ml) using water as eluent.

Nuclease K28C Variant. K28C was prepared by cassette mutagenesis, using a modified nuclease gene cloned into M13mp18 (30) as part of a screen for point mutants at residue 28. The mutation was confirmed by dideoxy sequencing, and the gene was subcloned into the pAS1 expression construct and transformed into the *Escherichia coli* AR120 expression strain. The protein was expressed and purified as described previously for wild-type nuclease (31). A final chromatographic step through Bio-Gel P30 (100–200 mesh, Bio-Rad) was added to remove traces of low molecular weight proteins. An HPLC map of a complete *Staphylococcus aureus* V8 protease digest of K28C showed only one peptide with anomalous retention time compared with a map of wild-type nuclease. Amino acid sequencing of that peptide confirmed the K28C substitution.

K28C-EDTA-Fe. K28C (60 nmol) was dissolved in 60 μl of 130 mM Tris·HCl (pH 7.2). EPD-Fe in water (120 nmol, 40 μl) was added, and the solution was incubated at room temperature for 1 hr. After the disulfide exchange reaction, the protein-EDTA-Fe conjugate was purified from residual EPD-Fe by spin-column chromatography (32). Nuclease was subjected to the same procedure and used as the control in the cleavage reactions.

Cleavage Reaction. Cleavage was carried out for 15 min at 4°C in 100 mM Tris·HCl (pH 7.2) and at 15 μM protein concentration. The reaction was initiated by adding ascorbate (adjusted to pH 7.2 with Tris) to give a final concentration of 20 mM. The reaction under denaturing conditions was performed after a 10-min preincubation with 0.1% SDS or 3 M guanidinium chloride at room temperature. In some control experiments, H_2O_2 was added (1 mM final concentration) immediately before ascorbate. Samples for SDS/PAGE analysis (120 μl) were frozen (−70°C), freeze-dried, dissolved in SDS/PAGE sample buffer, heated 10 min at 56°C, and electrophoresed. Samples for HPLC analysis were injected directly into the HPLC system to terminate the reaction.

RESULTS

The synthesis of EPD, **1**, is illustrated in Fig. 1. EDTA triethyl ester, **2**, was converted into cystamine-(EDTA triethyl ester)$_2$, **3**, upon carbodiimide-mediated coupling to cystamine. The ester **3** was hydrolyzed and the acid was converted to **1** *in situ* upon disulfide reduction and reaction

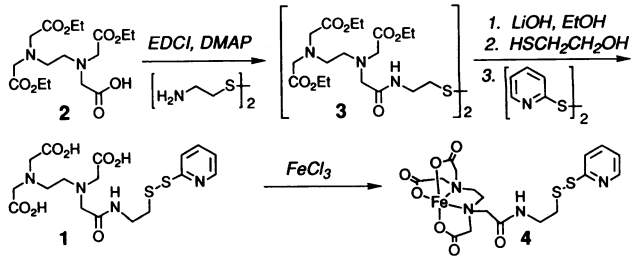

FIG. 1. The synthetic route to EPD and its Fe^{3+} complex. EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; DMAP, 4-dimethylaminopyridine.

with 2,2'-dipyridyl disulfide. The EPD–Fe complex, **4**, was obtained by incubating the lithium salt of EPD with $FeCl_3$.

The nuclease variant K28C (Lys-28 → Cys) was produced by cassette mutagenesis. The mutation was confirmed by dideoxy DNA sequencing. The expressed nuclease K28C was enzymatically active in a plate assay (33) and contained one cysteine residue per molecule as determined by amino acid analysis. The stabilities of K28C and wild-type nuclease (ΔG_D = 4.2 ± 0.3 kcal/mol and ΔG_D = 4.6 ± 0.1 kcal/mol, respectively) to guanidinium chloride-induced unfolding were not significantly different, as monitored by tryptophan fluorescence emission measurements. The single cysteine residue at position 28 was coupled specifically and quantitatively by disulfide exchange (29) to EPD–Fe, joining the EDTA–Fe complex to the protein through a disulfide bridge. The extent of the coupling was determined by measuring the residual free thiol content of the protein. Amino acid analysis also provided a simple and sensitive method to quantitate the degree of coupling, because performic acid oxidation (34) and acid hydrolysis (35) yield cysteic acid and taurine from the protein and attached chelate, respectively (taurine is eluted between cysteic and aspartic acid in conventional ion-exchange amino acid analysis). Iron determination showed that K28C–EDTA–Fe incorporated 0.9 mol of iron per mol of protein while, in control experiments, nuclease bound 0.1 mol of iron per mol of protein. The coupling reaction was also monitored by nondenaturing PAGE because nuclease, K28C, and K28C–EDTA–Fe all have distinct electrophoretic mobilities.

K28C–EDTA–Fe was subjected to cleavage reaction by incubation with 20 mM ascorbate for 15 min at 4°C. Approximately 75% of the aminoethyl disulfide group remained attached to the protein following the cleavage reaction as determined by amino acid analysis of the protein purified by HPLC. Reaction of native K28C–EDTA–Fe led to protein cleavage at multiple sites. SDS/PAGE analysis indicated that major products had apparent masses of 16 kDa (two bands), 14 kDa (one band), and 6–8 kDa (several bands) (Fig. 2 *A*, lane 3; *B*, lane 2; and *C*, lane 1). Controls without ascorbate (Fig. 2*A*, lane 1) and with wild-type nuclease (lacking cysteine residues and previously subjected to the EPD–Fe coupling procedure) (Fig. 2*A*, lane 6) showed that the reaction requires ascorbate and the covalently linked chelate. Nuclease was not cleaved by equimolar amounts of free EDTA–Fe or EPD–Fe (Fig. 2*B*, lanes 4 and 5), indicating that the cleavage is intramolecular. The reaction requires oxygen, as virtually no cleavage was observed under an argon atmosphere (data not shown). H_2O_2 was reported to dramatically increase the yield of the ascorbate-induced protein–EDTA–Fe cleavage with a related reagent (12). However, H_2O_2 produced only a modest increase in the extent of the ascorbate-induced K28C–EDTA–Fe cleavage in the present study (Fig. 2*A*, lane 4). H_2O_2 and ascorbate treatment of wild-type nuclease caused slight protein degradation (Fig. 2*A*, lane 7); therefore, H_2O_2 was not used further. The 6- to 8-kDa fragments produced in the cleavage reaction were isolated by HPLC (Fig. 3*A*, peaks a and b; Fig. 2*D*, lanes 1 and 2). These fragments were not observed when nuclease was incubated with ascorbate and free EPD–Fe or EDTA–Fe (Fig. 3 *D* and *E*) or when the cleavage reaction was performed in the presence of 3 M guanidinium chloride (Fig. 3*B*), confirming that the cleavage is intramolecular and also dependent upon the protein tertiary structure. The fragments of 16 and 14 kDa were coeluted with the uncleaved protein in the HPLC analysis (Fig. 3*A*, peak c; Fig. 2*D*, lane 3).

The 6- to 8-kDa products, purified by HPLC (Fig. 3*A*, peaks a and b) were submitted to sequence analysis. Cleav-

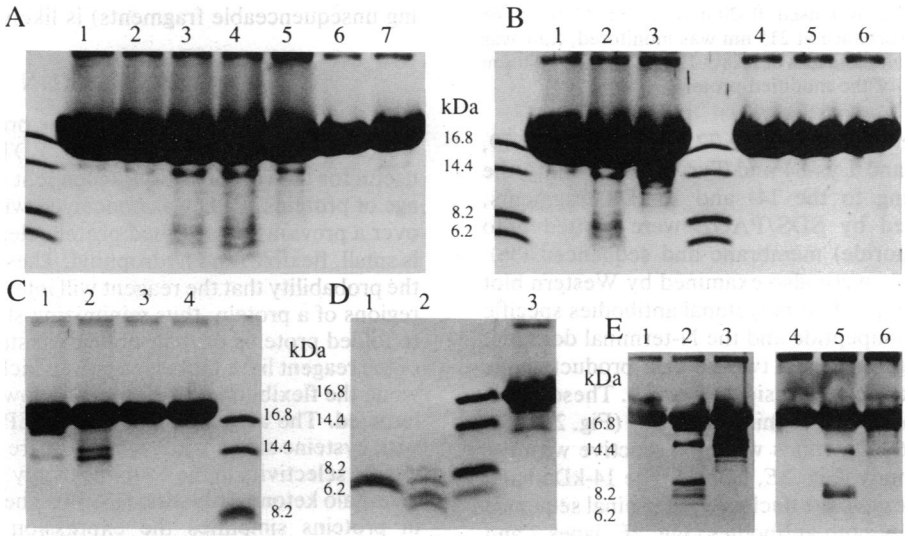

FIG. 2. SDS/PAGE analysis of the K28C–EDTA–Fe cleavage reaction products. The reaction was performed as described in *Materials and Methods*. Unless otherwise indicated, 40 μg of protein was loaded in each lane. Molecular size markers were run in unlabeled lanes. (*A*) K28C–EDTA–Fe incubated without ascorbate (lane 1); K28C–EDTA–Fe incubated without ascorbate, but ascorbate was included in the SDS/PAGE sample buffer (lane 2); K28C–EDTA–Fe and nuclease incubated with ascorbate (lanes 3 and 6, respectively); K28C–EDTA–Fe and nuclease incubated with both ascorbate and 1 mM H_2O_2 (lanes 4 and 7, respectively); K28C–EDTA–Fe incubated with 1 mM H_2O_2 without ascorbate (lane 5). (*B*) K28C–EDTA–Fe incubated without ascorbate (lane 1); K28C–EDTA–Fe incubated with ascorbate (lane 2); K28C–EDTA–Fe preincubated with 0.1% SDS (10 min at room temperature) before addition of ascorbate (lane 3); nuclease incubated with ascorbate (lane 4), with ascorbate and 15 μM EDTA–Fe (lane 5), and with ascorbate and 15 μM EPD–Fe (lane 6). (*C*) In order to better resolve the 14- to 18-kDa fragments, only 15 μg of protein was loaded in each lane and the electrophoresis was run longer. K28C–EDTA–Fe incubated with 20 mM ascorbate (lane 1) or preincubated with SDS and reacted with ascorbate (lane 2), with ascorbate and 15 mM EDTA–Fe (lane 3), and with ascorbate and 15 mM EPD–Fe (lane 4). (*D*) Cleavage reaction products isolated by HPLC (Fig. 3*A*): peaks a–c, lanes 1–3, respectively. (*E*) Western blot analysis of the cleavage reaction products. After SDS/PAGE analysis polypeptides were electroblotted to nitrocellulose sheets and immunostained as described in *Materials and Methods*. K28C–EDTA–Fe incubated without ascorbate (lanes 1 and 4), with ascorbate (lanes 2 and 5), and with ascorbate in the presence of 0.1% SDS (lanes 3 and 6). In lanes 1–3, detection was performed with an antibody specific for the C-terminal nonapeptide of nuclease. In lanes 4–6, an antibody specific for the N-terminal decapeptide of nuclease was used. Small quantities of nuclease dimer are present at the top of all lanes.

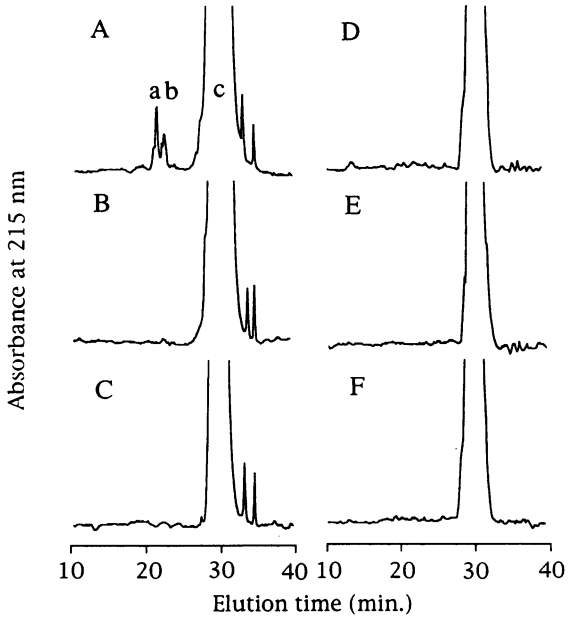

FIG. 3. HPLC analysis of the cleavage reaction products. K28C–EDTA–Fe and nuclease were subjected to the cleavage reaction. K28C–EDTA–Fe was reacted with ascorbate without additions (*A*), in the presence of 3 M guanidinium chloride (*B*), and without ascorbate (trace C). Wild-type nuclease was reacted with ascorbate in the presence of an equimolar amount of EPD–Fe (*D*) or EDTA–Fe (*E*) and without additions (*F*). Reactions were stopped by injecting the sample (120 μg of protein) into the HPLC column. Fractions a, b, and c (*A*) were collected and subjected to SDS/PAGE and sequence analysis. Chromatography was performed on a Vydac C_{18} column (4.6 × 250 mm). A linear gradient between solvents A (25% acetonitrile in 0.06% trifluoroacetic acid) and B (0.05% trifluoroacetic acid in acetonitrile) was used (0–20 min, 25–32.5% B; 20–68 min, 32.5–68% B). Absorbance at 215 nm was monitored; flow was 1 ml/min. Two minor contaminants of K28C–EDTA–Fe have elution times greater than that of the modified protein.

age occurred between Lys-71 and Ile-72, Lys-78 and Gly-79, Gln-80 and Arg-81, and Lys-84 and Tyr-85. To identify the cleavage sites leading to the 14- and 16-kDa fragments, polypeptides resolved by SDS/PAGE were blotted onto poly(vinylidene difluoride) membrane and sequenced (36). The cleavage products were also examined by Western blot analysis using affinity-purified polyclonal antibodies specific for the C-terminal nonapeptide and the N-terminal decapeptide of nuclease (Fig. 2*E*). The two 16-kDa products gave sequences starting at nuclease residues 1 and 9. These bands were reactive with the anti-C-terminal antibody (Fig. 2*E*, lane 2), and one of the 16-kDa bands was also reactive with the anti-N-terminal antibody (Fig. 2*E*, lane 5). The 14-kDa band from the same sample gave the nuclease N-terminal sequence and was recognized by both antibodies (Fig. 2*E*, lanes 2 and 5). Thus, under native conditions, K28C–EDTA–Fe was cleaved at residues 8, 71, 78, 80, and 84. Interestingly, two uncleaved products were also produced with altered electrophoretic mobility. Side-chain chemical modifications are the most likely explanation of this result: single side-chain modifications have been reported to alter the electrophoretic mobility of proteins on SDS/PAGE (37, 38).

The three-dimensional structure of wild-type nuclease and the relative positions of the cleavage points under native conditions are shown in Fig. 4. Cys-28 is located at the second position of a β-turn on the protein surface. All cleavage sites identified are near this turn. Three are located on a long surface loop, one on a surface β-strand, and the fifth in the short segment preceding the first β-strand in the molecule.

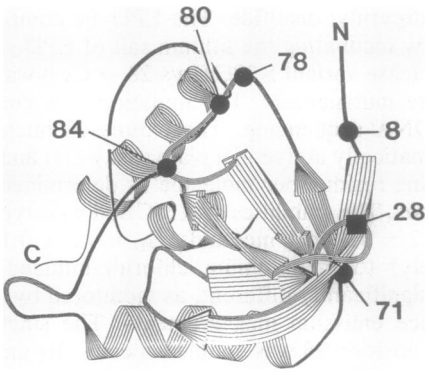

FIG. 4. Sites of polypeptide backbone cleavage under native conditions are identified (●) on a ribbon diagram of staphylococcal nuclease. Residue 28 is the site of EPD attachment (■).

The effect of the protein tertiary structure upon the cleavage reaction was examined by incubating K28C–EDTA–Fe with 0.1% SDS before the addition of ascorbate. In the presence of SDS, the two 16-kDa and the 6- to 8-kDa fragments (observed in cleavage of native protein, Fig. 2*B*, lane 2) were not produced. Instead, two heavy and several weak bands of 13–15 kDa were observed (Fig. 2 *B*, lane 3, and *C*, lane 2). Analysis of these products identified only a single sequence starting at Met-32. All of these fragments were reactive with the C-terminal antibody (Fig. 2*E*, lane 3), but only low levels of a 14-kDa band were detected with the anti-N-terminal antibody (Fig. 2*E*, lane 6). These results indicated that the SDS–protein complex of K28C–EDTA–Fe was cleaved between Pro-31 and Met-32 and at several other residues close to Cys-28. Since only one sequence was obtained, cleavage with α-amino modification (i.e., producing unsequenceable fragments) is likely to have occurred.

DISCUSSION

As part of an effort to understand the protein folding process, we designed and synthesized an EDTA derivative (EPD) useful for the conformation-dependent intramolecular cleavage of proteins. EPD was conceived with improved features over a previously described protein cleavage reagent (11). It is small, flexible, and hydrophilic. These properties diminish the probability that the reagent will interact with hydrophobic regions of a protein, thus minimizing structural perturbation to folded proteins or their nonnative states. The dimensions of the reagent limit the region over which cleavage can occur, while the flexibility of the reagent allows multiple sites to be targeted. The activated disulfide of EPD reacts exclusively with cysteine side chains, whereas care must be exercised to ensure selectivity in the coupling of any related reagent based on α-halo ketone activation (13, 39). The paucity of free thiols in proteins simplifies the expression of protein variants containing a single chelator attachment site located at a desired position. The EPD synthesis, protein modification procedure, and protein-EDTA adduct characterization are remarkably simple.

K28C–EDTA–Fe incubated 15 min at 4°C with ascorbate, under conditions chosen to promote a Fenton reaction (40, 41), was fragmented into several peptides. Examination of the nuclease crystal structure reveals that the cleavage sites identified are located close to Cys-28, the site of EPD attachment. These sites are near Cys-28 in the tertiary structure but as many as 56 residues away in the sequence. As expected, in the presence of denaturants, these sites are no longer attacked. Cleavage in an SDS solution leads to a different cleavage pattern, and only sites located close in the sequence to Cys-28 are attacked. Thus, EDTA–Fe cleavage

reagents may be utilized for the structural characterization of alternatively folded proteins.

Dissolved oxygen at atmospheric partial pressure and ascorbate are required for the cleavage reaction reported herein. K28C–EDTA–Fe was cleaved in both native and SDS-induced states. These results contrast with a previously reported cleavage reaction using a different protein-linked EDTA–Fe complex that required the addition of H_2O_2 and did not afford cleavage products in SDS solutions (11, 12). The cleavage mechanism proposed in that case involves a direct attack of a nucleophilic iron-peroxide group at the carbonyl carbon (13). Interestingly, a conformational search of the EDTA–Fe reagent attached to the nuclease crystal structure (20, 21) at position 28 indicates that the putative iron-peroxide cannot approach within van der Waals contact of the amide bonds cleaved (minimum distance, Fe–carbonyl: C_8, 8.9; C_{71}, 10.5; C_{78}, 10.4; C_{80}, 8.1; and C_{84}, 13.8 Å). Protein cleavage using the reagent described herein may involve reaction with diffusible oxygen species. Alternatively, direct attack of a reagent-bound iron-peroxide would be possible, if conformational fluctuations of the protein backbone occur, and would be consistent with cleavage products amenable to Edman degradation (13). The yield of cleavage products is lower and the number of cleavage products is greater with the EPD reagent than those observed with the previously described reagent (13). These differences may reflect a greater hydrophilicity and conformational flexibility of EPD and its highly solvent exposed site of attachment or a partial degradation of the EDTA chelate during the reaction.

This work describes the synthesis and evaluation of EPD as a stereospecific agent for protein cleavage. The observed properties of EPD suggest that, with the appropriate controls, the reagent could be a useful tool for characterizing proteins in nonnative folded states.

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