Oxidative Polypeptide Cleavage Mediated by EDTA–Fe Covalently Linked to Cysteine Residues†

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ABSTRACT: Chemical cleavage with reactive oxygen species generated by EPD–Fe, a protein-tethered EDTA–Fe reagent, has been proposed as a method to map the structure of nonnative equilibrium protein folding intermediates [Ermácora, M. R., Delfino, J. M., Cuenoud, B., Schepartz, A., & Fox, R. O. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89,* 6383–6387]. The chemical structure of protein cleavage products and the mechanism of backbone scission for this class of reagents have been unclear. Here, we report the nature of EPD–Fe-mediated backbone cleavage of a small model peptide. The EPD–Fe reagent was attached to a partially α-helical peptide, α1BAla (Ac-AEAEEAAKKAKEACKA-NH_2), through a mixed disulfide. Backbone cleavage was initiated by addition of the iron reductant ascorbate. Chemical analysis of the novel cleavage products revealed an oxidative cleavage mechanism, probably initiated by diffusible hydroxyl radicals. The EPD–Fe-mediated cleavage technique appears to be suitable for the analysis of nonnative protein states such as the molten globule.

Nonnative protein folding intermediates can be stabilized at equilibrium and have been partially characterized by NMR[1] under favorable circumstances (Kim & Baldwin, 1990; Dobson et al., 1991). Recently, we proposed the use of a chemical cleavage technique to investigate the structure of these nonnative states (Ermácora et al., 1992). In this method, an EDTA–Fe reagent (EPD–Fe) is attached through a mixed disulfide to a cysteine residue of a protein. Upon addition of the iron reductant ascorbate, reactive oxygen species are formed, resulting in protein backbone cleavage at surface sites in the vicinity of the reagent (Ermácora et al., 1992). Sequencing of the resulting products has been used to identify residues that have been brought close to the site of attachment by the fold of the protein and that are accessible to cleavage-promoting reactive oxygen species. The technique has been used to identify residues on the surface of staphylococcal nuclease in the native and nonnative states (M. R. Ermácora, D. W. Ledman, H. W. Hellinga, G. S. Hsu, and R. O. Fox, manuscript in preparation).

The EDTA–Fe-mediated formation of hydroxyl radicals has been used extensively in footprinting protein–DNA complexes (Dervan, 1986; Tullius, 1989). The region of DNA in proximity to a particular site on the DNA-binding domain of γδ resolvase was mapped by covalently attaching EDTA to a peptide during solid-phase synthesis and carrying out DNA cleavage in the complex (Dervan, 1986). Indeed, EPD has been used to map protein–DNA interactions for γδ resolvase (Mazzarelli et al., 1993), the *Escherichia coli* catabolite gene activator protein (CAP), and the λ cro protein (Ebright et al., 1992).

Protein cleavage by reactive oxygen species is well-known (Garrison, 1987). EDTA–Fe covalently attached to protein ligands has been used in the affinity cleavage of proteins (Hoyer et al., 1990; Schepartz & Cuenoud, 1990). Rana and Meares (1990, 1991a) reported the first cysteine-specific EDTA–Fe derivative (BABE) that could be covalently attached to a protein via an α-bromo ketone and that mediates cleavage of the protein backbone in a conformation-dependent manner. The BABE reagent was proposed to cleave the polypeptide backbone by the nucleophilic attack of a reagent-bound metal peroxo species, and this proposal was supported by a series of mechanistic studies (Rana & Meares, 1991b). Diffusible hydroxyl radicals have also been implicated in an oxidative cleavage mechanism in a related system, leading to a distinct set of cleavage products (Hoyer et al., 1990).

Ermácora et al. (1992) developed the EPD reagent in the hope that it would be more suitable than BABE for the analysis of nonnative protein states. EPD is more hydrophilic and more flexible than BABE and yields a greater array of cleavage products. The cleavage yield observed using EPD is lower than that for BABE, but is sufficient for chemical analysis of the cleavage products.

The nature of the cleavage mechanism could have a considerable impact on the suitability of a reagent in the analysis of dynamic nonnative protein states. A stable iron–peroxo species might oversample compact, native-like states if its lifetime is greater than that of the equilibrium folding intermediates under investigation. The highly reactive oxygen radicals presumably have shorter lifetimes than those of folding intermediates and would be expected to provide a more realistic view of a partially folded structure.

Here we examine in detail the EDTA–Fe-mediated cleavage products of a 16-residue, partially α-helical peptide, α1BAla, modified with EPD–Fe to investigate the cleavage mechanism of the EPD–Fe reagent and to examine the role of conformation in the distribution of cleavage sites.

MATERIALS AND METHODS

General Details. All chemicals were reagent grade and were used without further purification. HPLC was carried out with a computer-controlled system (Rabbit HP, Rainin)

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[1] Abbreviations: BABE, 1-[*p*-(bromoacetamido)benzyl]-EDTA; BME, β-mercaptoethanol; CD, circular dichroism; DTT, *threo*-1,4-dimercapto-2,3-butanediol; EDTA, ethylenediaminetetraacetic acid; EPD, EDTA-2-aminoethyl 2-pyridyl disulfide; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; NEM, *N*-ethylmaleimide; NMR, nuclear magnetic resonance; PAO, performic acid oxidation; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid.

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and a Vydac (C_{18}, 4.6 × 250 mm) column. A gradient between solvent A (water with 0.06% TFA) and solvent B (0.05% TFA in acetonitrile) was used (0–10 min 0% B; 10–60 min, 0–40% B). Absorbance at 215 nm was monitored, and the flow was 1.0 mL/min.

Circular dichroism (CD) spectra were obtained with an Aviv Circular Dichroism DS-60 spectropolarimeter. Mass spectra were recorded using a VG ZAB-SE high-resolution spectrometer with fast atom bombardment (FAB-cesium) ion source. Thiol groups were assayed using Ellman's reagent (Ellman, 1957). Performic acid oxidation was performed as described (Hirs, 1967). Amino acid analyses were determined with a 7300 Beckman Autoanalyzer. Peptides were sequenced using an Applied Biosystems 470A sequencer.

Synthesis of α1BAla. The 16-residue α1BAla peptide, Ac-AEAEEAAKKAKEACKA-NH_2, was synthesized on an Applied Biosystems Inc. Model 430A solid-phase synthesizer using standard *tert*-butyloxycarbonyl chemistry and HF deblocking cleavage. This peptide is a modified version of α1B (Ho & DeGrado, 1987), which showed high α-helical content by CD in aqueous solutions. The modifications introduced were the following: All leucine and glycine residues were replaced by alanine, and a cysteine residue was placed at position 14. α1BAla was purified by reverse-phase HPLC (C_{18} column), and its chemical structure was confirmed by analytical HPLC, amino acid analysis, and mass spectrometry. HPLC analysis and Ellman's reaction revealed that more than 97% of the peptide was in its reduced form and had one free thiol group per molecule.

Synthesis of EPD. The synthesis was performed as previously described (Ermácora et al., 1992).

Synthesis of [α1BAla-S-]$_2$. α1BAla (0.25 mM in 100 mM Tris-HCl, pH 8.0) was allowed to oxidize in air at 20 °C for 7.5 h. The disulfide-linked dimer was purified from reduced starting material by reverse-phase HPLC.

Synthesis of α1BAla–EDTA–Fe. EPD and α1BAla, both at 0.5 mM final concentration in 100 mM Tris-HCl (pH 7.2), were reacted for 3 h at 20 °C. The peptide–EDTA adduct was isolated by reverse-phase HPLC. The structure of α1BAla–EDTA was confirmed by mass spectrometry [FAB-MS *m/e* 2094.3 (MH^+ calcd 2094, 3-nitrobenzyl alcohol/CF_3CO_2H matrix)] and amino acid analysis after performic acid oxidation. The latter procedure generated, as expected, a 1:1 taurine:cysteic acid molar ratio (Ermácora et al., 1992). $FeCl_3 \cdot H_2O$ was added to a 1.0 mM solution of α1BAla–EDTA in 100 mM Tris-HCl (pH 7.2) to give a 1:1 Fe^{3+}:peptide ratio. The solution was incubated for 20 min at 20 °C and used in the cleavage reaction.

Cleavage Reaction. α1BAla–EDTA–Fe solutions (1 mM) were incubated with 20 mM ascorbate at 20 °C for 50 min. Ascorbate was added as a freshly prepared 200 mM solution, which had been adjusted to pH 7.2 with Tris base just before use. The reaction was terminated by injection into the HPLC system. The following controls were also performed: (a) ascorbate was omitted; (b) α1BAla (the peptide with the free thiol group) or α1BAla–NEM (the peptide with the thiol group blocked by reaction with *N*-ethylmaleimide) was incubated with free EDTA–Fe or free EPD–Fe, respectively; (c) α1BAla–EDTA was substituted for α1BAla–EDTA–Fe; (d) the standard reaction was terminated by reduction with an excess of either 2-mercaptoethanol (4% final solution, 1.5 h, 60 °C) or *threo*-1,4-dimercapto-2,3-butanediol (DTT) (1 M, 3 h, 62 °C) and then injected into the HPLC system.

Circular Dichroism Experiments. α1BAla and α1BAla–EDTA–Fe were dissolved in 100 mM potassium fluoride at pH 7.2. Peptide concentration was determined by amino acid analysis. Ellipticity as a function of wavelength was measured in the range of 200–300 nm. The molar ellipticity per residue was calculated by the formula, $[\theta] = 100\theta/(Cnl)$ where θ is ellipticity in degrees, C is the molar peptide concentration, l is the path length in centimeters, and n is the number of residues.

RESULTS

Design of the α1BAla sequence was based on the α1B peptide that associates to form a four α-helix bundle structure (Ho & DeGrado, 1987). The Leu and Gly residues of α1B were substituted with Ala with the intention of maintaining a partially α-helical structure with an uncharged Ala face that would not associate to form an oligomer. Many monodisperse α-helical peptides have been synthesized with similar amino acid compositions (Chakrabarity et al., 1991). Cys-14 was introduced as the site of reagent attachment because it falls above an α-helical face of Ala residues used as targets for the cleavage reaction when the α1BAla peptide is modeled in an α-helical conformation. The EDTA–Fe also projects from its site of attachment near the C-terminus toward the N-terminus at a point near the middle of the Ala face in the α-helical model. The small size of the α1BAla peptide and its helix–coil conformational equilibrium made it an attractive system to characterize the EPD–Fe-mediated cleavage products and reaction mechanisms in greater detail than had previously been possible with a protein (Ermácora et al., 1992).

The α1BAla peptide was easily modified with the cysteine-specific EPD reagent and complexed with Fe^{3+} as described in Materials and Methods and outlined in Figure 1. The secondary structures of the α1BAla peptide and the α1BAla–EDTA–Fe adduct were examined using CD spectroscopy (Figure 2). The α1BAla–EDTA–Fe adduct displayed a CD spectrum that was indicative of a partially α-helical conformation. EPD modification did not appear to significantly perturb the peptide conformation. We estimate that the α1BAla peptide has a 40% average α-helicity based on $[\theta_{222}]$ = 34 400 for a fully α-helical peptide of this size (Chen et al., 1974). Circular dichroism does not indicate whether the α-helicity is an average of molecules in full helix and coil or the result of molecules that are always in a partially helical conformation. The extent of α-helicity of the α1BAla–EDTA–Fe adduct could be reduced by the addition of the denaturant guanidinium hydrochloride and increasing temperature, as evidenced by the progressive loss of the CD signal at $[\theta_{222}]$ (data not shown). The CD spectrum of the α1BAla–EDTA–Fe was invariant at concentrations ranging from 0.32 to 32.1 μM (not shown), in contrast to the strong concentration dependence of $[\theta_{222}]$ observed for the related, but more hydrophobic, α1B peptide (Ho & DeGrado, 1987). The α1BAla peptide is thus unlikely to associate over the concentration range examined.

The α1BAla–EDTA–Fe cleavage reaction was initiated by the addition of ascorbate (as described in Materials and Methods) and characterized by reverse-phase HPLC as shown in Figure 3a. No significant reaction was observed without the addition of ascorbate (Figure 3b), as previously shown for staphylococcal nuclease cleavage using the EPD reagent (Ermácora et al., 1992). No cleavage was observed if equimolar amounts of free EDTA–Fe and α1BAla peptide were incubated with ascorbate under the standard reaction conditions. Likewise, when the Cys-14 sulfhydryl of α1BAla is blocked by NEM, no cleavage products were observed when

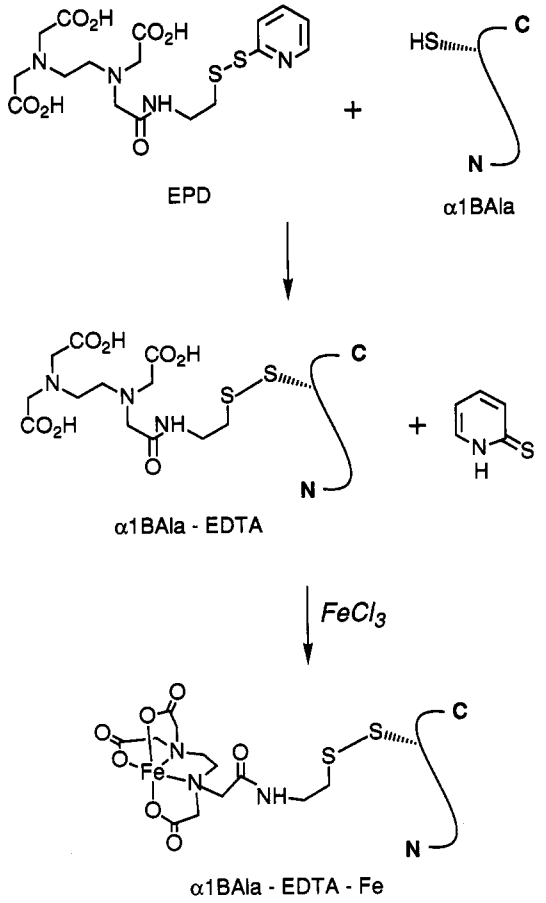

FIGURE 1: α1BAla peptide modified at Cys-14 with the EPD reagent through disulfide exchange. The iron complex of the modified peptide was prepared by the addition of $FeCl_3$ before the cleavage reaction was initiated with ascorbate.

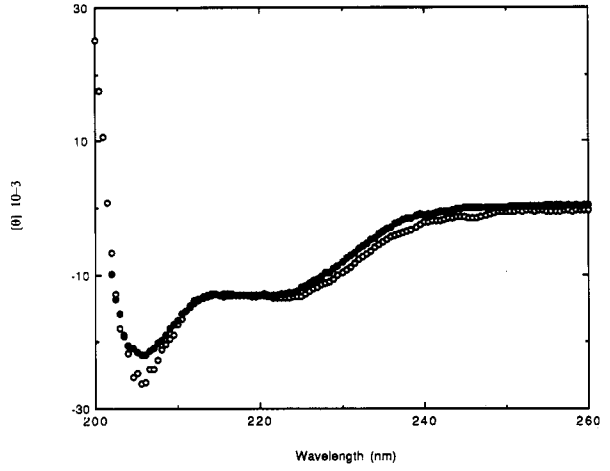

FIGURE 2: Circular dichroism spectra of α1BAla (○) and α1BAla–EDTA–Fe (●). Measurements were made at 22 °C in 100 mM KF buffer in a 1 cm path length cell. The peptide concentrations were determined by amino acid analysis and used to calculate the molar elipticity, [θ].

the blocked peptide was incubated with an equimolar amount of EPD–Fe under standard reaction conditions. Thus, the reaction products observed in Figure 3a are the result of an intramolecular reaction of the α1BAla–EDTA–Fe adduct.

Oxygen was required for the protein cleavage experiment involving EPD (Ermácora et al., 1992). In the present experiments, a decrease in yield coincided with a decrease in the surface-to-volume ratio of the solution, an observation consistent with the requirement of continued O_2 consumption

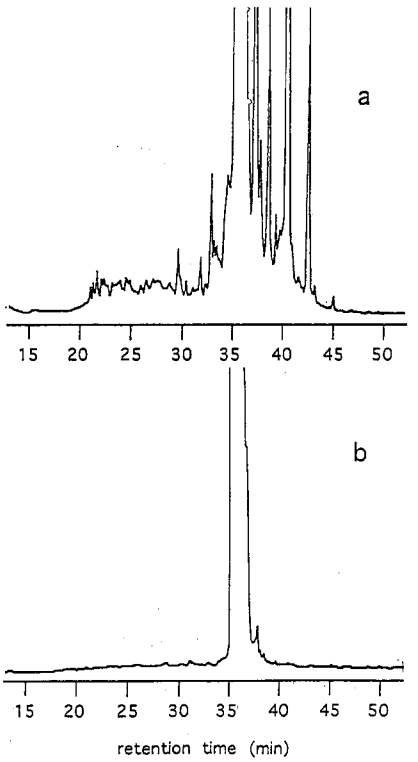

FIGURE 3: Reverse-phase C_{18} HPLC traces of α1BAla–EDTA–Fe (a) following the reaction and (b) without the addition of ascorbate. The absorbance was modified at 215 nm and is reported in arbitrary units, but both panels are on a common scale. Peaks in a correspond to the retention times in Table I.

to support this reaction. The cleavage product yield also fell off sharply as the temperature was increased above 60 °C, resulting in almost no cleavage at 80 °C (Platis, 1992). This may reflect the decreased solubility of O_2 at these temperatures.

The ascorbate-mediated reaction of α1BAla–EDTA–Fe resulted in a complex mixture of products (Figure 3a), of which many, but not all, were characterized by mass spectrometry, amino acid analysis, or sequence determination. Reduction of specific products with BME or DTT followed by HPLC or mass spectrometry aided in identifying two EDTA adducts and oxidized forms of the Cys-14 sulfhydryl group. A number of reaction products did not yield a mass spectrum on repeated trials with sufficient material. These products were subjected to performic acid oxidation and amino acid analysis. When a cysteic acid residue was present, indicating a C-terminal peptide fragment, the peptide was sequenced. The analytical data for each reaction product characterized are presented in Table I. The retention times cited are those from the chromatogram in Figure 3a. The results of amino acid analysis and sequencing are presented in Tables I and II.

Twelve different cleavage products and a number of chemically modified peptides were isolated and characterized (Tables I and II). The masses and amino acid compositions of three fragments (**10**, **12**, and **13**) were not consistent with any single or double cleavage product of the α1BAla peptide on the basis of a hydrolytic scission of the peptide bond (Figures 4b and 5A) (Rana & Meares, 1991b). They also were not compatible with the previously identified peptide oxidative cleavage products (Figure 4c) (Hoyer et al., 1990). Mass calculations were carried out with and without the attached reagent, or observed degradation products of the reagent, and oxidation states of the Cys-14 sulfhydryl group. None of the calculated masses matched those observed for **10**, **12**, and **13**. We were thus forced to consider alternative chemical structures

Table I

compd	t_R[a] (min)	[M + H]$^+$ obsd	[M + H]$^+$ calcd	amino acid analysis[b] A	C	E	K	X[d]	assignment[c]
1	21.3			3.0(3)	1.0(1)	1.2(1)	1.9(2)		10–16
2	21.6			3.0(3)	1.0(1)	1.2(1)	2.6(3)		9–16
3	21.9			3.1(3)	1.0(1)	1.1(1)	3.3(4)		8–16
4	23.3								6–16
5	24.9								5–16
6	26.3			4.9(5)	1.0(1)	3.1(3)	3.6(4)		4–16
7	26.9			6.1(6)	1.0(1)	3.1(3)	3.6(4)		3–16
8	27.8								2–16
9	29.7			4.1(4)		2.9(3)	1.0(1)	1.2(1)	1–9RCO[e]
10	31.8	1029.4	1029.5	3.8(4)		2.4(2)	2.4(2)	ND[f]	1–10RCO[e]
11	33.0	2019.6	2019.9						1–16EDTA*[g]
12	33.5	1287.1	1286.7	5.2(5)		3.0(3)	2.9(3)	ND[f]	1–12RCO[e]
13	34.5	1358.0	1357.7	no pure peptide was isolated					1–13RCO[e]
14	36.6	2091.8	2090.8	7.3(7)	1.0(1)	4.2(4)	4.1(4)		1–16EDTAFe
15	37.3	1961.2	1961.9						1–16EDTA*[g]
16	37.8	1703.9	1704.9						1–16SOH[h]
17	38.8	1689.1	1688.9	6.9(7)	1.0(1)	4.1(4)	4.1(4)		1–16
18	40.4	3376.9	3374.7	14(14)	2.0(2)	8.2(8)	7.8(8)		$[1–16]_2$
19	42.4	1720.6	1720.8	7.0(7)	0.9(1)	4.2(4)	4.0(4)		1–16SO_2H[i]

[a] Retention time in HPLC analysis. [b] Ion exchange amino acid analysis with ninhydrin detection after acid hydrolysis. Cysteine was determined as cysteic acid after performic acid oxidation. [c] Mass determination, amino acid analysis and, if available, sequence data (see Table II) were used to determine the chemical structure of the products. [d] Oxidation at the α-carbon of lysine residues, followed by backbone cleavage yielded 5-aminovaleric acid. This product was detected by conventional amino acid analysis. The same cleavage at glutamic acid and at alanine residues would result in succinic acid and acetic acid, respectively. Both derivatives are ninhydrin-negative. [e] The C-terminus of the peptide is an oxidation product with the following general structure: NHRCO. [f] Not detected. [g] Oxidative degradation of the EDTA moiety. [h] Sulfenic acid derivative of α1BAla. [i] Sulfinic acid derivative of α1BAla.

Table II [a]

peptide sequence	MS	compd
Ac-A-E-A-E-E-A-A-K-K-A-K-E-A-C-K-A-amide	+	α1BAla
Ac-A-E-A-E-E-A-A-K-K-A*	+	10
Ac-A-E-A-E-E-A-A-K-K-A-K-E*	+	12
Ac-A-E-A-E-E-A-A-K-K-A-K-E-A*	+	13
Ac-A-E-A-E-E-A-A-K-K*	+[b]	9
A-K-E-A-C-K-A-amide		1
K-A-K-E-A-C-K-A-amide		2
K-K-A-K-E-A-C-K-A-amide		3
A-A-K-K-A-K-E-A-C-K-A-amide		4
E-A-A-K-K-A-K-E-A-C-K-A-amide		5
E-E-A-A-K-K-A-K-E-A-C-K-A-amide		6
A-E-E-A-A-K-K-A-K-F-A-C-K-A-amide		7
E-A-E-E-A-A-K-K-A-K-E-A-C-K-A-amide		8

[a] Peptides identified by mass spectroscopy are indicated by a plus in the MS column. Peptides confirmed by amino acid analysis (Table I) are underlined. Sequences identified by amino acid sequencing are shown in boldface type. [b] The mass of **9** was confirmed by electrospray MS. An asterisk indicates the presence of a modified C-terminal residue as illustrated in Figure 4d.

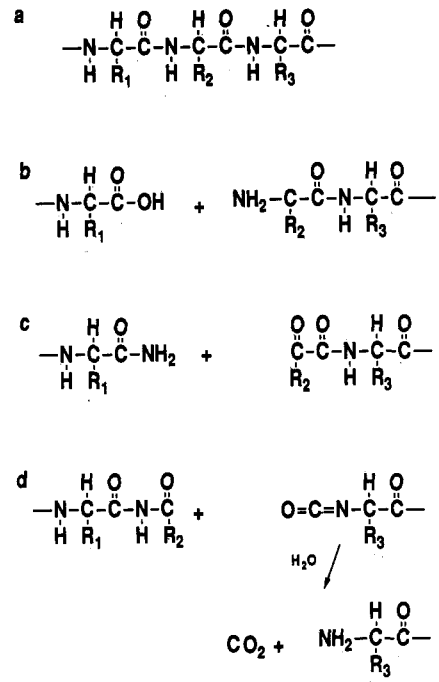

FIGURE 4: Cleavage products of a peptide segment (a) resulting from (b) hydrolytic cleavage, as proposed by Rana and Meares (1991b), (c) the oxidative mechanism cited by Hoyer et al. (1990), and (d) the mechanism proposed to lead to the oxidative products observed herein.

for these cleavage products. Amino acid analysis of these products identified them as N-terminal fragments. The C-terminal residue of one of these fragments is oxidized to a carbonyl group at the α-carbon position with the loss of the α-carboxyl group and the residues later in the sequence (Figure 4d). The masses calculated for the chemical structures of fragments **10**, **12**, and **13** are in agreement with the observed masses within the expected error of <1 mass unit (Table I). The amino acid composition determined for **9** is consistent with the proposed chemical structure (including 1 equiv of 5-aminovaleric acid expected from the acid hydrolysis of the modified Lys-9 residue). Peptides **10** and **13**, with modified C-terminal Ala residues, should yield acetic acid on hydrolysis, while peptide **12** should yield succinic acid from its modified Glu C-terminus. The succinic and acetic acids could not be determined by amino acid analysis. The sequences of these fragments were not investigated given the acetylated N-terminus of α1BAla.

A number of peptides did not yield FAB-MS (**1–9**). Amino acid analysis of each performic acid oxidized product for **1**,

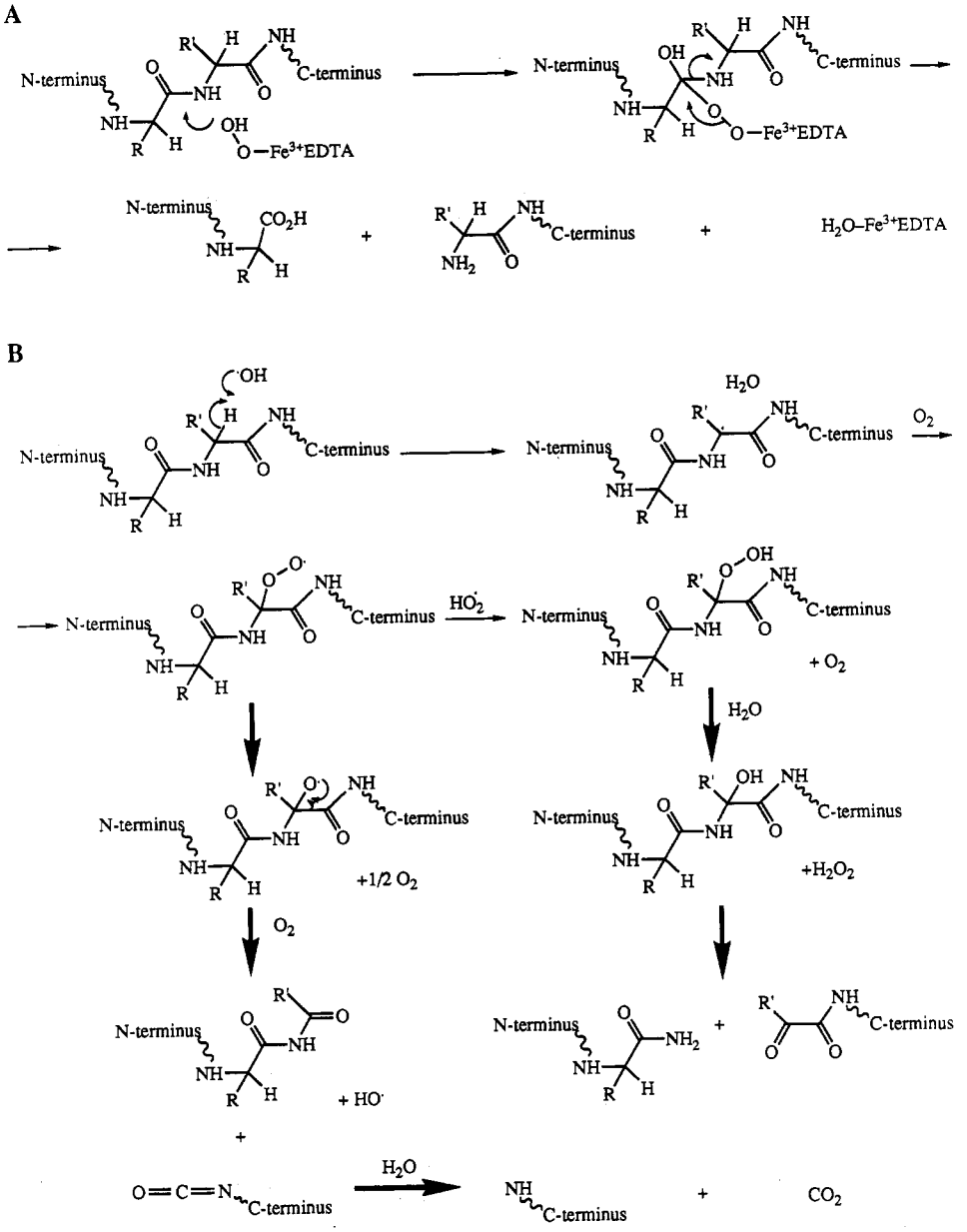

FIGURE 5: Mechanisms of polypeptide backbone cleavage: (A) hydrolytic cleavage mechanism mediated by an EDTA–Fe–peroxo species, as proposed by Rana and Meares (1991b); (B) proposed cleavage of the polypeptide backbone mediated by hydroxyl radical abstraction of the $C_\alpha H$ hydride and subsequent recombination with oxygen. Decomposition of this adduct along the path to the right leads to the products reported by Hoyer et al. (1990) (Figure 4c), while breakdown along the pathway to the left leads to the products reported herein (Figure 4d).

2, **3**, **6**, and **7** yielded cysteic acid, indicating that these fragments were derived from the C-terminal portion of the peptide. Edman sequencing of the peptides (without PAO treatment) revealed that they had a free N-terminus and a unique sequence identifying their chemical structure (Table II). The modified Cys-14 residue did not give an interpretable PTH product and was assumed to remain as an EDTA–Fe adduct. The sequence of **7** was not carried to completion, but the information obtained is compatible with only one single cleavage product.

A number of reaction products of the α1BAla–EDTA–Fe adduct did not involve cleavage of the peptide backbone. The major products involved cleavage of the disulfide bridge between the reagent and the peptide, resulting in free α1BAla (**17**) and the sulfinic (**19**) and sulfenic (**16**) oxidation states of Cys-14. These forms were identified by mass spectrometry. Reduction of **16** and **19** with DTT to the α1BAla peptide (**17**) was confirmed by mass spectrometry as expected for sulfinic and sulfenic acids (Houghten & Li, 1979). The amino acid compositions for α1BAla and **16** were confirmed by PAO and amino acid analysis. The Cys-14 sulfhydryl group was oxidized to the sulfonic acid (α1BAla–SO_3) by PAO and characterized by HPLC and mass spectrometry. The retention time did not match those of any of the reaction products. The peptide dimer [α1BAla–S–]$_2$ (**18**) was identified by mass spectrometry and by the similarity of its HPLC retention time to that of the synthetic dimer (see Materials and Methods). The dimer is presumed to form by the combination of α1BAla sylfhydryl radicals (α1BAla–S·) formed during the reaction.

Two chemical modifications to the reagent were observed (**11** and **15**). Reduction of these products resulted in α1BAla (**17**) (determined by its HPLC retention time), indicating a modification to the EDTA moiety. The mass of **11** was consistent with the loss of an oxygen atom from the reagent and can be explained by a variety of cyclized EDTA derivatives. The mass of **15** is consistent with the loss of a carboxymethylene group and an oxygen atom and is also consistent with a distinct set of cyclized EDTA derivatives.

Under the experimental conditions used, a significant fraction of α1BAla–EDTA–Fe remains uncleaved (~61%). Cleavage of the disulfide bond is the most frequent chemical event, yielding free α1BAla (**17**, ~2%) and, on oxidation, the sulfinic (**19**, ~3%) and sulfenic (**16**, ~2%) acids and the disulfide-linked dimer form of the peptide (**18**, ~11%). Two chemical modifications of the reagent occurred at relatively low yields: ~2% **11** and ~1% **15**. The remainder of the α1BAla–EDTA–Fe peptide adduct (~18%) was cleaved along the peptide backbone. The lower yield and complexity of the cleavage mixture made it difficult to determine the yield of individual cleavage products; however, they appear to range from 0.5% to 2%. No oxidative modifications to Ala, Lys, or Glu side chains were observed. Also, no cleavage products were observed that could only be explained by two cleavage events.

DISCUSSION

Cleavage was observed almost uniformly over the length of the α1BAla peptide (residues 2–13). Cleavage occurring at the remaining residues may not have been detected due to the HPLC behavior of the products. If the α1BAla peptide formed a stable α-helical conformation throughout its length, the reagent would be positioned over the Ala residues and generally away from the Glu and Lys residues. The partial α-helical character of the peptide did not strongly bias the pattern of cleavage observed toward the Ala residues, although minor variations in yield were not estimated. The uniform cleavage may be due to the partially unfolded state of the peptide and the short lifetime of an α-helix, allowing access to all peptide units in each molecule during the lifetime of the active oxygen species (hydroxyl radicals). The greater disorder expected at the ends of α-helices may also contribute to an increased conformational range of the reagent when bound to the peptide, allowing it access to all surfaces of the helix and coil conformations. Unlike an α-helix on the surface of a protein, the α1BAla peptide is uniformly exposed to solvent and potentially accessible to diffusible hydroxyl radicals. These results are in contrast to those obtained for staphylococcal nuclease, where there is a strong correlation between the solvent-accessible protein surface and the observed cleavage sites (Ermácora et al., 1992; M. R. Ermácora, D. W. Ledman, H. W. Hellinga, G. S. Hsu, and R. O. Fox, manuscript in preparation).

The uniform cleavage observed for the α1BAla peptide indicates a lack of bias in the cleavage due to side-chain identity (for Ala, Lys, and Glu) and rules out the specific need for side-chain participation in the cleavage reaction. The uniform cleavage also shows the expected distribution of products for a flexible, solvent-exposed peptide, even in the presence of partial α-helical structure. Thus, biases for and against particular residues in the folded state must arise from their differential accessibility to solvent (Ermácora et al., 1992).

The peptide cleavage products reported herein are consistent with the general chemical forms shown in Figure 4d, and with the mechanism outlined in Figure 5B. Cleavage products of this type and the proposed mechanism were first observed in protein radiolysis experiments (Garrison, 1987). The cleavage reaction is initiated by the hydroxyl radical abstraction of a $C_\alpha H$ hydride leaving a C_α radical. The C_α radical can combine with a variety of oxygen species, resulting in an array of unstable adducts. These C_α-modified peptides can break down with cleavage of the NH–C_α or C_α–C(O) bond. Cleavage of the NH–C_α bond yields products of the type shown in Figure 4c. The resulting C-terminal fragment has a blocked N-terminal and should not yield sequence by Edman degradation. The complementary N-terminal fragment is amidated at the C-terminal residue. Cleavage of the C_α–C(O) bond yields products as in Figure 4d. The C-terminal fragment is initially formed as the corresponding isocyanate, which decomposes to yield a free amino terminus suitable for sequencing by Edman degradation. The N-terminal fragment has a carbonyl group at the C_α-position of the C-terminal residue. Hydrolysis of this material results in the C_α-acid of the modified C-terminal residue. Identification of such a product (5-aminovaleric acid) after acid hydrolysis of **9** and electrospray mass spectrometry confirmed cleavage by the latter mechanism at the Lys-9 residue. This type of cleavage product was also confirmed for **10**, **12**, and **13** by mass spectrometry.

The difficulty in characterizing a number of the cleavage products by FAB-MS led us to investigate HPLC electrospray MS methods (A. W. Guzzetta, W. S. Hancock, M. R. Ermácora, I. E. Platis, and R. O. Fox, unpublished results). The HPLC–ESI–MS method confirmed the MS data presented herein, but also indicated that cleavage products from the other oxidative mechanism (Figure 4c) and the hydrolytic mechanism (Figure 4b) were also present. A full account of these results will be presented in a future communication. Thus, a number of polypeptide cleavage mechanisms must coexist, mediated by the tricarboxylate EPD reagent, in contrast to the single hydrolytic mechanism observed for the tetracarboxylate BABE reagent (Rana & Meares, 1991b). This may result from differences in the Fe^{3+} coordination of BABE and EPD, such that BABE may stabilize an iron–peroxo species to a greater extent. If hydroxyl radicals are produced at a lower rate by BABE, the reagent may be more stable and lead to higher protein cleavage yields.

A large fraction of the α1BAla–EDTA–Fe adduct is dissociated under the cleavage reaction conditions by oxidative cleavage of the disulfide bond. This results in free peptide, the oxidized sulfinic and sulfenic acid states of the α1BAla Cys-14 sulfhydryl group, and α1BAla disulfide-linked dimers. Two types of reagent modification have also been inferred from the mass spectrometry data. At the α1BAla–EDTA–Fe concentrations used, intermolecular cleavage reactions between EDTA–Fe or EPD–Fe and peptide do not occur. Thus once the reagent is removed from the peptide, no further cleavage occurs. Together, these properties of the EPD–Fe system result in a relatively low cleavage yield. This is a favorable situation in the study of protein structure where a single cleavage event can profoundly change the folded state or structure of a protein. Because the disulfide linkage is much more susceptible to oxidative cleavage than a peptide bond, and once dissociated the reagent is unable to cause cleavage, the EPD–Fe system favors a single backbone cleavage event per molecule. This simplifies both the analysis and the interpretation of cleavage data from peptides and proteins. The yield of cleavage products is relatively low, but is sufficient for amino acid analysis, Edman degradation, and mass spectrometry. The low yields and the large number of cleavage products combine to make quantitation difficult.

In conclusion, the chemistry of the EPD–Fe system appears favorable for the study of nonnative protein states such as the molten globule. EPD–Fe-mediated cleavage of the polypeptide backbone is oxidative, probably initiated by diffusible and highly reactive hydroxyl radicals. The cleavage reaction shows little sequence selectivity among the residues examined (Ala, Lys, and Glu) and extends over a range of about 10 residues from the site of attachment in this flexible system. The lifetime of the reactive oxygen species involved is probably shorter than that of the protein folding intermediates (though probably

not that of an α-helix) and thus should provide a reasonable map of the accessible surface of nonnative protein structures.

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