Articles

Protein Interactions Leading to Conformational Changes Monitored by Limited Proteolysis: Apo Form and Fragments of Horse Cytochrome *c*†

Barbara Spolaore, Ruperto Bermejo,‡ Marcello Zambonin, and Angelo Fontana*

CRIBI Biotechnology Centre, University of Padua, Viale G. Colombo 3, I-35121 Padua, Italy

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ABSTRACT: Proteolysis experiments have been used to monitor the conformational transitions from an unfolded to a folded state occurring when the apo form of horse cytochrome *c* (cyt *c*) binds the heme moiety or when two fragments of cyt *c* form a native-like 1:1 complex. Proteinase K was used as a proteolytic probe, in view of the fact that the broad substrate specificity of this protease allows digestion at many sites along a polypeptide chain. The rather unfolded apo form of cyt *c* binds heme with a concomitant conformational transition to a folded species characterized by an enhanced content of helical secondary structure. While the holoprotein is fully resistant to proteolytic digestion and the apoprotein is digested to small peptides, the noncovalent complex of the apoprotein and heme exhibits an intermediate resistance to proteolysis, in agreement with the fact that the more folded structure of the complex makes the protein substrate more resistant to proteolysis. The noncovalent native-like complex of the two fragments 1−56 and 57−104 of cyt *c*, covering the entire polypeptide chain of 104 residues of the protein, is rather resistant to proteolysis, while the individual fragments are easily digested. Fragment 57−104 is fast degraded to several peptides, while fragment 1−56 is slowly degraded stepwise from its C-terminal end, leading initially mostly to fragments 1−48 and 1−40 and, at later stages of proteolysis, fragments 1−38, 1−35, 1−33, and 1−31. Thus, proteolysis data indicate that the heme containing fragment 1−56 has a rather compact core and a C-terminal flexible tail. Upon prolonged incubation of the complex of fragments 1−56 and 57−104 (nicked cyt *c*) with proteinase K, a chain segment is removed from the nicked protein, leading to a gapped protein complex of fragments of 1−48 and 57−104 and, on further digestion, fragments 1−40 and 57−104. Of interest, the chain segment being removed by proteolysis of the complex matches the ω-loop which is evolutionarily removed in cyt *c* of microbial origin. Overall, rates and/or resistance to proteolysis correlates well with the extent of folding of the protein substrates, as deduced from circular dichroism measurements. Thus, our results underscore the utility of proteolytic probes for analyzing conformational and dynamic features of proteins. Finally, a specific interest of the cyt *c* fragment system herewith investigated resides in the fact that the fragments are exactly the exon products of the cyt *c* gene.

A variety of spectroscopic techniques is available for monitoring conformational transitions of polypeptides in solution (*1*), the most commonly used being circular dichroism (CD)[1] (*2*, *3*). Far-UV CD measurements allow evaluation of overall features of the secondary structure of proteins, as well as quantification of the relative proportions of α-helix, β-sheet, and random coil (*4*, *5*). Tertiary structure and the microenvironment of aromatic chromophores in a protein can be monitored by near-UV CD (*6*, *7*), fluorescence emission and quenching (*8*−*10*), and differential absorption in the UV region (*11*, *12*). Also, infrared (*13*) and Raman (*14*, *15*) spectroscopy can be used to estimate conformational features and transitions of proteins. At present, the most useful and informative technique for the structural elucidation of (small) proteins in solution is NMR spectroscopy (*16*, *17*). However, NMR usually remains plagued by heavy instrumentation requirements, needs a millimolar concentration of a nonaggregating protein solution, and, in addition, is of limited success in the detailed analysis of partly folded and fluctuating states of proteins due to resonance broadening and/or a lack of sufficient chemical shift dispersion (*18*, *19*). Even X-ray crystallography can be utilized for protein structure analysis only if suitable protein crystals are available, and

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* To whom correspondence should be addressed: CRIBI Biotechnology Centre, University of Padua, Viale G. Colombo 3, I-35121 Padua, Italy. Telephone: +39-049-8276156. Fax: +39-049-8276159. E-mail: fontana@civ.bio.unipd.it.

‡ Present address: Department of Physical and Analytical Chemistry, University of Jaen, EUP of Linares, Linares 23700, Spain.

[1] Abbreviations: cyt *c*, cytochrome *c*; apoCyt, apo form of cytochrome *c*; complex of fragments 1−56 and 57−104, 1:1 mixture of fragments 1−56 and 57−104 of cyt *c*; CD, circular dichroism; SDS, sodium dodecyl sulfate; [θ], mean residue ellipticity; MALDI, matrix-assisted laser-desorption ionization; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse-phase high-performance liquid chromatography; Tricine, *N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine; TFA, trifluoroacetic acid; TFE, trifluoroethanol; DTT, dithiothreitol.

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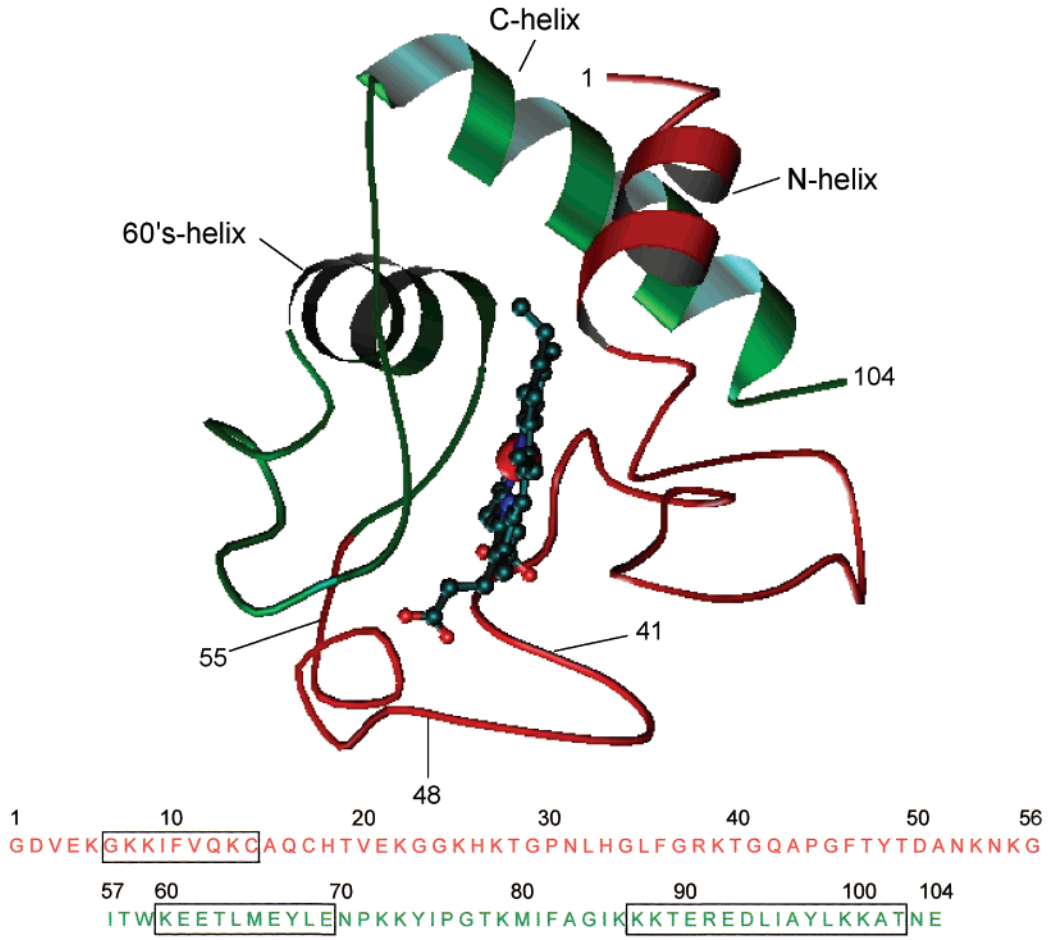

FIGURE 1: Three-dimensional structure (top) and amino acid sequence (bottom) of horse heart cytochrome *c*. The three major helices of the protein are shown as a ribbon, and the remaining residues are represented by a string. The heme group, covalently bound to Cys14 and Cys17 via thioether bridges, is shown as a ball-and-stick representation. Numbers near the polypeptide backbone indicate the location of the amino acid residues relevant to the discussion of the results of this study. Fragments 1−56 and 57−104 are shown in red and green, respectively. The model was constructed from Brookhaven Protein Data Bank X-ray structure 1HRC (*54*) using the program MolScript (*89*). The three major helices (N-, C-, and 60's-helix) are boxed in the amino acid sequence of the protein (bottom).

moreover, it is of no use with dynamic protein systems. Therefore, no one technique is fully superior to all others, and each one has advantages and drawbacks.

Limited proteolysis proved to be a valuable tool for probing conformational features of proteins (*20*−*27*). This approach relies on the fact that proteolysis of a protein substrate can occur only if the polypeptide chain can bind and adapt to the specific stereochemistry of the protease's active site (*28*). This is difficult to achieve with native rigid protein structures, whereas unfolded proteins are degraded much faster. Nevertheless, even native globular proteins can be attacked by a protease, and in a number of cases, it has been shown that the peptide bond fission occurs only at one peptide bond or a few peptide bonds (*20*, *25*, *27*, *29*). In previous studies, we have emphasized that the key feature of the chain sites or regions prone to limited proteolysis resides in their enhanced flexibility, as given by the fact that there is a correlation between sites of limited proteolysis and sites of enhanced segmental mobility, this last measured by the crystallographic temperature factors (*B*-values) (*21*). Indeed, it has been shown that limited proteolysis occurs at the level of flexible loops, outside regions embedded in a rigid, hydrogen-bonded secondary structure (such as α-helices) (*21*−*24*, *29*). Therefore, proteolytic probes can be used both to monitor overall conformational transitions of a polypeptide chain and to pinpoint along the chain sites of local unfolding (*25*−*27*, *29*).

Here, limited proteolysis experiments have been used to monitor the folding of a polypeptide chain from a rather unstructured state to a folded, helical state. For these experiments, we utilized the apo form of horse cytochrome *c* (cyt *c*) and its N- and C-terminal fragments comprising residues 1−56 and 57−104, respectively (see Figure 1). It is shown that the folding of the polypeptide chain, as given by far-UV CD measurements, resulting from the binding of heme to apocytochrome *c* (apoCyt) (*30*, *31*), as well as from the association of the two fragments into a folded native-like complex, can be monitored by using proteinase K as a proteolytic probe. Thus, our results show that the simple biochemical technique of limited proteolysis can provide useful protein structural data, complementing those obtained by the commonly used CD technique.[2]

MATERIALS AND METHODS

Materials. Horse heart cytochrome *c* (type VI), proteinase K, and hemin were obtained from Sigma. Cyanogen bromide (BrCN), dithiothreitol (DTT), acetonitrile, and trifluoroacetic acid (TFA) were purchased from Fluka, and the materials used for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were from Bio-Rad. All other

chemicals were analytical grade and were obtained from Sigma or Fluka.

Apocytochrome *c* (apoCyt) was prepared by Ag^+-mediated removal of the heme from cyt *c* following essentially the procedure previously described (*31*). Stock solutions of apoCyt were prepared by dissolving the lyophilized protein in distilled water at a concentration of ~0.1 mM and stored frozen at −20 °C.

The N-terminal heme-fragment 1−56 and C-terminal fragment 57−104 were prepared by limited proteolysis of cyt *c* with thermolysin in aqueous trifluoroethanol (TFE) (*32*). Digestion was performed at 25 °C with cyt *c* dissolved (1 mg/mL) in 10 mM Tris-HCl/5 mM $CaCl_2$ buffer (pH 7.8) in the presence of 50% (v/v) TFE and using a protease: protein substrate ratio of 1:50 (by mass). The reaction was stopped after 6 h with aqueous TFA (final TFA concentration of 0.1%). The fragments were then separated by gel filtration chromatography utilizing a column (2.6 cm × 95 cm) prepared with Sephadex G-50 superfine, which was equilibrated and eluted with 5% (v/v) aqueous formic acid. The homogeneity of N- and C-terminal fragments 1−56 and 57−104, respectively, was determined by RP-HPLC and SDS−PAGE and their identity established by mass spectrometry.

Proteolysis Experiments. Proteolysis of apoCyt, cyt *c*, and the apoCyt−heme complex by proteinase K was conducted at 25 °C by dissolving (~0.4 mg/mL) the protein or its complex with heme in 0.1 M phosphate buffer (pH 7.7) containing 10 mM DTT. The apoCyt−heme complex was obtained at a heme:apoCyt molar ratio of 1.1:1. Proteolysis of fragment 1−56 or 57−104 of cyt *c* by proteinase K was performed in 10 mM phosphate buffer (pH 7.1) at a fragment concentration of 130 μM. The digestion of the complex of fragments 1−56 and 57−104 was conducted in 10 mM phosphate buffer (pH 7.1) using a 65 μM solution of both fragments (1:1 molar ratio). Proteolysis was always performed using a proteinase K:substrate (E:S) ratio of 1:100 (by mass). At intervals, aliquots were taken from the reaction mixtures, and the proteolysis was stopped by adding 2% TFA. Samples were concentrated using the Speed-Vac system (Savant) and then analyzed by SDS−PAGE, RP-HPLC, and MALDI mass spectrometry. For some HPLC-purified protein fragments, N-terminal sequencing was also performed utilizing an Applied Biosystems model 477A protein sequencer.

Electrophoresis and Chromatography. SDS−PAGE was carried out in a vertical slab gel apparatus (Mini-Protean II, Bio-Rad) using the Tricine buffer system (*33*). The gels were stained with Coomassie brilliant blue R-250. A sample of a partial BrCN cleavage of horse cyt *c* at the level of the two methionine residues at positions 65 and 80 of the 104-residue chain of the protein (*34*) was used as the molecular mass standard. The resulting fragments are made up of residues 1−80, 1−65, 66−104, and 81−104 (in the order of decreasing molecular mass); the small fragment (66−80) does not appear in the Coomassie-stained SDS−PAGE gel. The BrCN reaction does not cleave off the heme moiety covalently bound to the polypeptide chain via two thioether bridges involving Cys14 and Cys17, and therefore, fragments 1−80 and 1−65 contain the heme.

Reverse-phase HPLC was performed utilizing a Vydac C_4 column (4.6 mm × 150 mm) purchased from The Separations Group. Elution was carried out at a flow rate of 0.8 mL/min with a linear gradient of a water/acetonitrile mixture containing 0.05% (v/v) TFA from 5 to 50% acetonitrile over the course of 30 min. The effluent was monitored by measuring the absorbance at 226 nm.

Mass Spectrometry. Mass spectra were recorded using a time-of-flight matrix-assisted laser-desorption ionization (MALDI) mass spectrometer (Kompact MALDI-I, Kratos-Shimadzu, Manchester, U.K.). α-Cyano-4-hydroxycinnamic acid dissolved in acetonitrile and 0.1% aqueous TFA (2:3 ratio, v/v) was used as a matrix, while bovine insulin and synthetic peptides of known molecular masses were used as standards for instrument calibration. Raw data were analyzed by the program Kompact provided by Kratos. The digestion mixtures of fragment 1−56 or 57−104 and of the complex of fragments 1−56 and 57−104 were diluted 10-fold (from 130 to 13 μM) with 0.1% aqueous TFA to optimize the fragment concentration required for MALDI mass spectral analysis.

Spectroscopic Measurements. Protein and fragment concentrations were determined spectrophotometrically using a Perkin-Elmer Lambda-20 spectrophotometer. The concentrations of stock solutions of holo cyt *c* and heme-fragment 1−56 were determined from their Soret absorbance (*35*, *36*) while those of the apo form of cyt *c* and fragment 57−104 from their absorbance at 280 nm according to Gill and von Hippel (*37*). The concentration of hemin was determined using an extinction coefficient of 5.84×10^4 M^{-1} cm^{-1} at 385 nm in 0.1 N NaOH (*30*, *31*). Circular dichroism (CD) measurements were taken at 25 °C on a Jasco (Tokyo, Japan) J-710 spectropolarimeter equipped with a thermostatically controlled cell holder. A 1 mm path length quartz cell was used for measurements, and the mean residue ellipticity [θ] is expressed in units of deg cm^2 $dmol^{-1}$ (for other experimental details, see ref *32*). Fluorescence emission measurements were performed using a Perkin-Elmer model LS-50 spectrofluorimeter using a cuvette with a path length of 1 cm.

RESULTS

Heme−Apocytochrome c Interactions. Apocytochrome *c* (apoCyt), the protein devoid of heme, at neutral pH appears to be largely unfolded on the basis of its far-UV CD spectrum (Figure 2A), indicating that the heme moiety is needed to achieve the folded state of the native protein. However, the features of the CD spectrum are not exactly those expected for a fully unfolded species (*2*−*5*), since the dichroic signal at 220 nm is -3500 ± 200 deg cm^2 $dmol^{-1}$, whereas in the presence of 6 M guanidine hydrochloride, this figure is -500 ± 200 deg cm^2 $dmol^{-1}$ (not shown). As previously reported by others (*30*, *31*), the binding of heme to apoCyt leads to the formation of a noncovalent complex and to a significant enhancement of the negative ellipticity in the region of 210−240 nm (Figure 2A). The overall features of the CD spectrum with minima at ~208 and ~220 nm are those of helical polypeptides (*4*, *5*). Nevertheless, the content of secondary structure of the heme−apoCyt complex is still lower than

[2] This work was presented at the Meeting of the Italian Protein Society, June 16−18, 2000, Varese, Italy [(2000) *Italian Biochemical Society Transactions 14*, A31], and at the Fourth European Symposium of the Protein Society, April 18−22, 2001, Paris, France [(2001) *Protein Sci. 10* (Suppl. 1), 171].

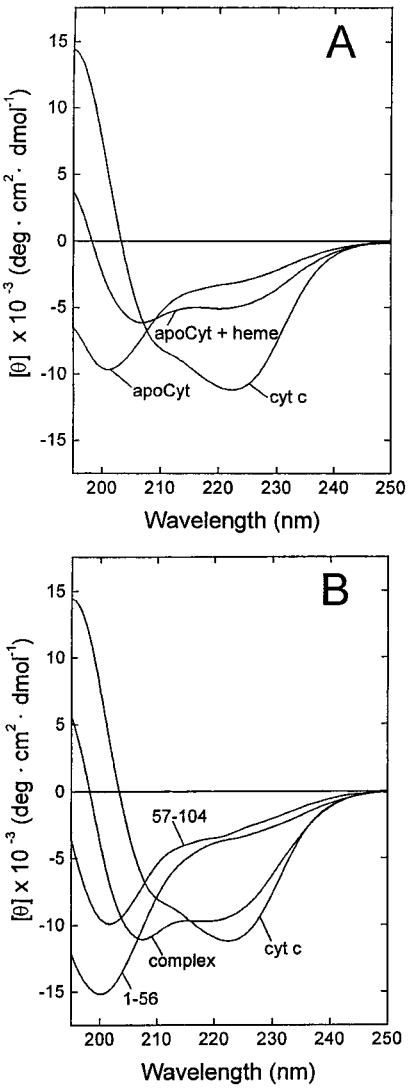

FIGURE 2: (A) Far-UV CD spectra of apoCyt, the apoCyt−heme complex, and cyt *c* in 0.1 M phosphate buffer (pH 7.5). (B) Far-UV CD spectra of cyt *c*, fragment 1−56, fragment 57−104, and the complex of fragments 1−56 and 57−104 in 10 mM phosphate buffer (pH 7.0).

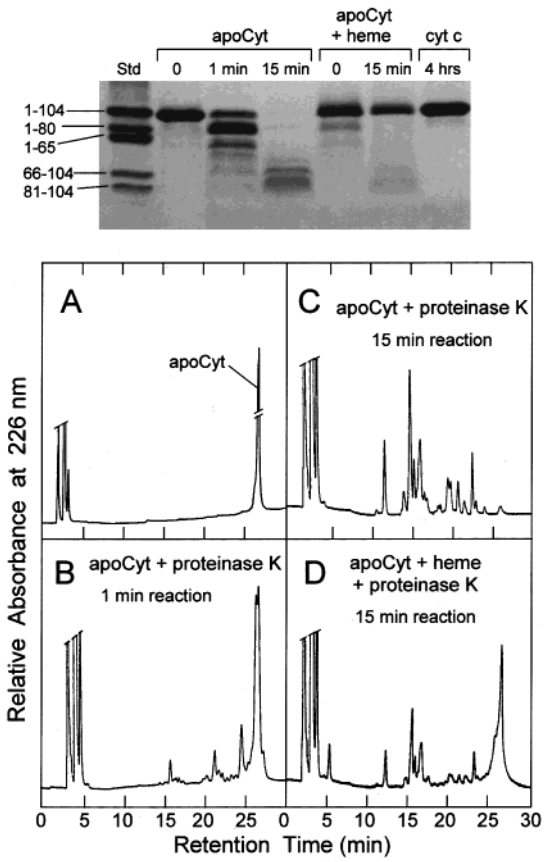

FIGURE 3: (Top) Analysis by SDS−PAGE of the proteolysis of apoCyt, the apoCyt−heme complex, and cyt *c* with proteinase K in 0.1 M phosphate buffer (pH 7.7). The protein was digested at a protease-to-substrate ratio of 1:100 (by mass) at 25 °C. The BrCN fragments of cyt *c* (see Materials and Methods) served as molecular mass markers and are shown on the left. (Bottom) RP-HPLC analysis of apoCyt (A), the proteolytic mixture of apoCyt (B and C), and the apoCyt−heme complex (D) with proteinase K. A Vydac C_4 column (4.6 mm × 150 mm) was employed.

that of the native holoprotein (see Figure 2A).

To monitor the heme-induced conformational transition of apoCyt, proteinase K was used as a proteolytic probe, since this is a voracious and nonspecific protease (*38*). Therefore, proteolysis of a protein substrate by proteinase K is expected to be dictated by the conformational and dynamic features of the protein substrate and not by its amino acid sequence. Figure 3 shows the results of SDS−PAGE and RP-HPLC analyses of the proteolytic mixtures of apoCyt without or with heme added to the reaction mixture. It is seen that proteolysis is rather fast in the absence of heme so that after reaction for 15 min the apoCyt substrate is fully digested. On the other hand, upon addition of 1.1 equiv of heme to the apoCyt solution and thus formation of the folded apoCyt−heme complex (*30*, *31*) (see above), the digestion is much retarded, apoCyt being still present in the reaction mixture, as shown by both SDS−PAGE and RP-HPLC data (Figure 3). Conversely, holo cyt *c* is highly resistant to proteolysis when reacted under identical conditions, and even after reaction for 4 h, the cyt *c* protein band in the stained SDS−PAGE gel is seen.

Although proteolysis of apoCyt in the absence of heme is very fast (see the top of Figure 3), the pattern of protein breakdown at the initial stages of the reaction (1 min) is not fully random along the polypeptide chain and thus leading to many small peptides, since a set of protein bands of relatively large molecular mass are formed (Figure 3, top, lane 3). The proteolytic mixture obtained after reaction for 1 min was analyzed by RP-HPLC (Figure 3, bottom, part B), and the identity of the major fragments eluted from the RP column was established by mass spectrometry and N-terminal sequencing (data not shown). The results of these analyses allowed us to identify the rather large fragments (19−79, 19−97, 19−98, and 19−104) as those formed at the initial stages of proteolysis of apoCyt at the level of the His18−Thr19, Lys79−Met80, Tyr97−Leu98, and Leu98−Lys99 peptide bonds (see Figure 1 for the amino acid sequence of cyt *c*). Therefore, proteolysis data appear to indicate that apoCyt retains some residual structure that renders regions of the polypeptide chain somewhat resistant to proteolysis. Evidence for residual structure in apoCyt is also provided by far-UV CD data (see above and Figure 2A). An estimate of an ∼5% helical content in apoCyt can be obtained from the far-UV CD spectrum of the protein (*2*, *4*). However, there are severe limitations in quantitating the helix content from far-UV CD spectra when the intensity of

the dichroic signal near 220 nm is weak (*4*, *5*).

Noncovalent Complex Given by the Association of Fragments 1−56 and 57−104. N- and C-terminal cyt *c* fragments 1−56 and 57−104, respectively, have been prepared previously in our laboratory by selective proteolysis of the holoprotein dissolved in 50% (v/v) aqueous trifluoroethanol (TFE) with thermolysin (*32*). The relative facility with which these thermolytic fragments, covering the entire polypeptide chain of the protein, can be prepared and their key feature of being exactly the protein fragments corresponding to the exons of the cyt *c* gene (*39*−*41*) (see also, Discussion), prompted us to examine the possibility that they are able to associate into a noncovalent complex, thus producing a complex given by fragments 1−56 and 57−104 (nicked cyt *c*). This was plausible, considering that previously several cyt *c* fragments were shown to associate in a stable complex characterized by some native-like characteristics (*35*, *36*, *42*−*45*).

As shown in Figure 2B, N-terminal heme-fragment 1−56 and C-terminal fragment 57−104 in isolation do not attain significant amounts of regular secondary structure, since the far-UV CD spectra are those of an unfolded polypeptide, even though not fully devoid of structure (see above the comments for apoCyt). On the other hand, the far-UV CD spectrum of the 1:1 mixture of the fragments shows the characteristics of α-helical polypeptide and is quite similar to that of intact cyt *c*, even if the ratio of ellipticities at 208 and 220 nm is different (Figure 2B). Therefore, these CD data indicate that the two fragments combine to form a folded complex, in analogy to other fragment complementing systems of horse (*42*, *43*) or tuna (*44*) cyt *c*. The conformational features of the complex of fragments 1−56 and 57−104 were further analyzed by CD spectroscopy in the near-UV and Soret region, UV absorption, fluorescence emission, and size-exclusion chromatography. These data, presented in the Supporting Information, allow us to conclude that the complex, or nicked cyt *c*, adopts an overall fold similar to that of native holo cyt *c*, but that the fission of the Gly56−Ile57 peptide bond leads to a more dynamic protein entity.

Proteolysis by proteinase K of the individual fragments in isolation, as well as in their complex state, was used to ascertain their conformational features. Figure 4 shows the SDS−PAGE and RP-HPLC analyses of the various proteolytic mixtures, while in Figure 5, the corresponding kinetic analyses by MALDI-MS are shown. The results of these analyses should be viewed as complementing each other and providing analytical data characterized by different levels of precision and detail of the same reactions. The comparative evaluation of these data allows us to derive the following conclusions. The isolated fragments do not resist proteolysis, and after reaction for 30 min, both are fully missing from the proteolytic mixtures. However, while fragment 57−104 leads to a variety of relatively small peptides only weakly visible in the Coomassie-stained gel (Figure 4, top, lane 5), fragment 1−56 instead is digested to a medium-size peptide fragment (Figure 4, top, lane 3). This last fragment was shown to comprise mostly residues 1−40 of cyt *c* on the basis of the MS analysis. Clearly, the MS data of Figure 5A indicate that fragment 1−56, upon prolonged digestion, is stepwise degraded from its C-terminal end, leading to peptides 1−48, 1−40, 1−38, 1−35, 1−33, and 1−31.

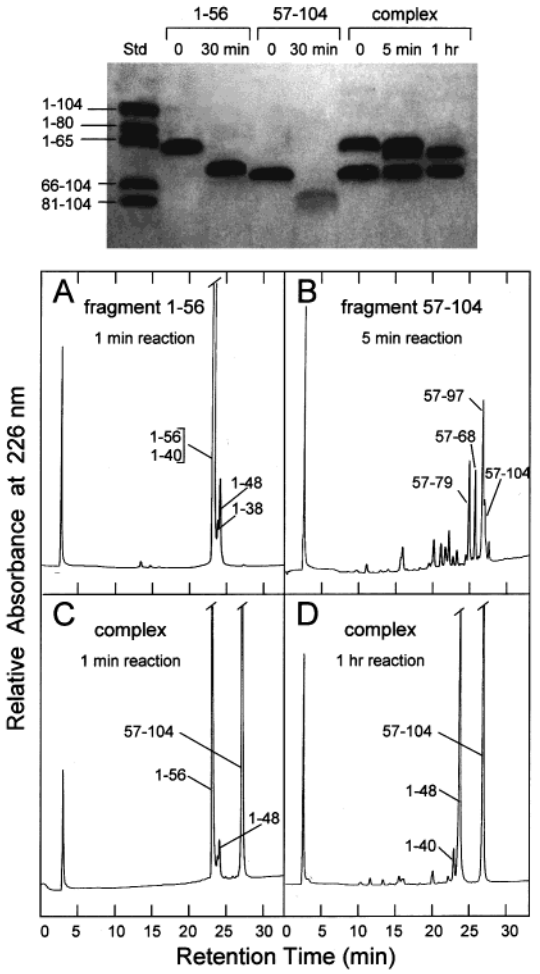

FIGURE 4: (Top) SDS−PAGE of the proteolytic mixtures of fragments 1−56 and 57−104 and of the complex of fragments 1−56 and 57−104 with proteinase K. The reactions were performed at 25 °C with a protease-to-substrate ratio of 1:100 (by mass). Numbers near the electrophoretic bands indicate the BrCN fragments of cyt *c*. (Bottom) RP-HPLC of fragments 1−56 (A) and 57−104 (B) and of the complex of fragments 1−56 and 57−104 (C and D) reacted with proteinase K at different reaction times. The experimental conditions for RP-HPLC were the same as those described in the legend of Figure 3. Numbers near chromatographic peaks refer to the identity of the fragments eluted from the column. Fragment identification was performed by N-terminal sequencing and MALDI-MS (see Materials and Methods).

The noncovalent complex of fragments 1−56 and 57−104 (nicked cyt *c*) is significantly more resistant to proteolysis than its constituent fragments in isolation. The key result derived from the SDS−PAGE and RP-HPLC data shown in Figure 4, combined with the MALDI-MS data of Figure 5, is that fragment 57−104, which is highly prone to proteolytic degradation in isolation (see above), becomes fully resistant to proteolysis if embedded in the complex of fragments 1−56 and 57−104. This is clearly demonstrated by both SDS−PAGE and RP-HPLC data, as well as by the fact that in the MALDI-MS spectrogram of the proteolytic mixture there are no fragments originating from the degradation of fragment 57−104 (see Figure 5C). On the other hand, in the complex of fragments 1−56 and 57−104, N-terminal fragment 1−56 is susceptible to a slow proteolysis and is somewhat stepwise degraded by proteinase K from its C-terminus, leading mostly to fragment 1−48 after reaction for 1 h. A much longer reaction time (5 h) leads to an

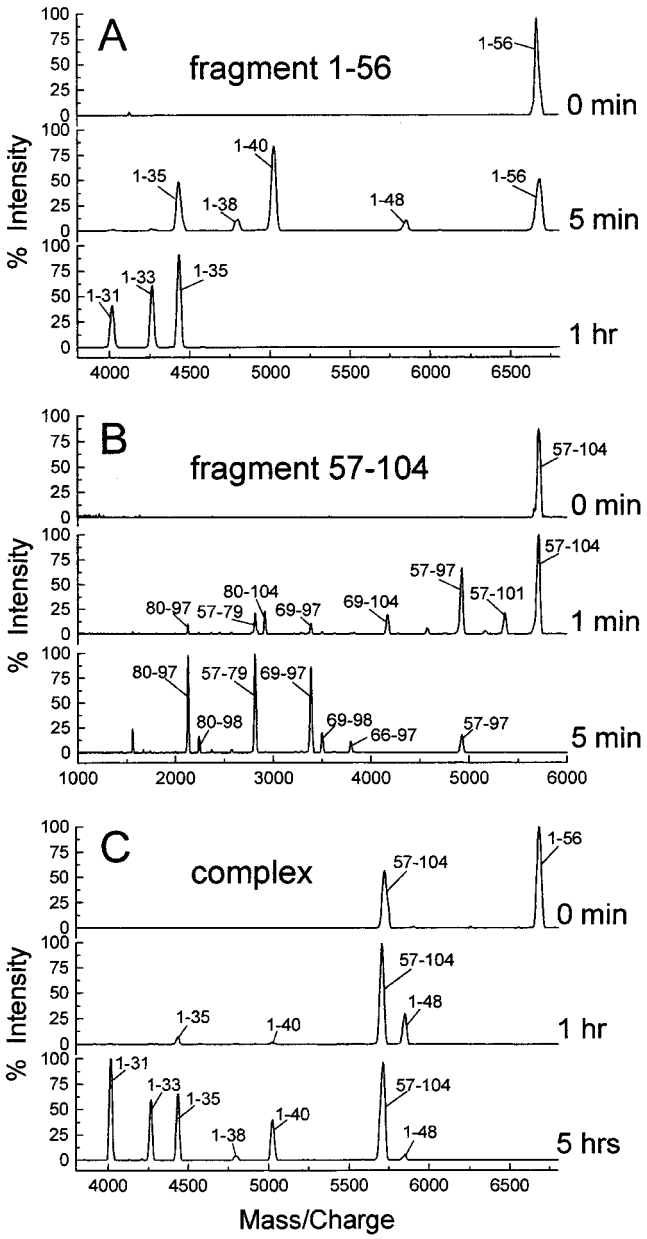

FIGURE 5: Time course analysis of the proteolysis of (A) fragment 1−56, (B) fragment 57−104, and (C) their complex by MALDI mass spectrometry. Numbers near the MS peaks refer to the identity of the fragments of cyt *c*. The identity of the peaks in the MALDI-MS spectrum was established by matching the measured mass values with the theoretical ones calculated from the amino acid sequence of cyt *c*.

N-terminal core fragment with a ragged C-terminal end (from residue 31 to 48), in analogy to isolated fragment 1−56. In conclusion, the core of the complex of fragments 1−56 and 57−104 is rather stable to proteolysis, while the chain segment from residue 48 to 56 in the complex appears to be flexible enough to be easily and selectively excised from nicked cyt *c*, leaving in solution mostly a gapped cyt c species given by fragments 1−48 and 57−104.

DISCUSSION

In this work, we aimed to demonstrate that proteolytic probes can be used to monitor conformational transitions of proteins. To test this possibility, we have chosen the structural organization occurring when apoCyt binds exogenously added heme, as well as when two protein fragments of cyt *c* combine in a 1:1 complex. In both cases, the protein system in its initial state is largely unfolded and acquires a compact and helical state upon binding a ligand or by fragment complementation. The heme-induced conformational transition in apoCyt has been studied before by spectroscopy (*30*) and immunochemical methods (*31*). Fragment complementing systems of cyt *c* have also been studied previously (*42*−*45*), but the specific interest of the system investigated herewith resides in the fact that fragments 1−56 and 57−104 correspond exactly to the exon products of the cyt *c* gene (*39*−*41*) (see also below). First of all, the conformational transitions of apoCyt upon addition of the heme prosthetic group and of the cyt *c* fragments upon association have been monitored by the most classical spectroscopic technique widely employed in protein structure analyses, i.e., far-UV CD (*3*−*5*). In agreement with previous studies (*30*), while the CD spectrum of apoCyt is that of a mostly unfolded polypeptide chain (*46*, *47*), the spectrum of the apoCyt−heme complex instead shows the characteristics of helical polypeptides with minima of ellipticity near 210 and 220 nm, even if the spectrum is quite different from that of native cyt *c* (see Figure 2A). Previously, it has been found that the apoCyt chain binds about two heme moieties and that this complex adopts a structure that is almost as compact as that of holo cyt *c* (*30*, *31*). Similarly, while isolated fragments 1−56 and 57−104 appear to be rather unstructured by CD criteria, the complex of fragments 1−56 and 57−104 exhibits a far-UV CD spectrum quite similar to that of intact cyt *c* (see Figure 2B), thus indicating that the fragments associate to form a complex. Since the far-UV CD spectrum of the complex of fragments 1−56 and 57−104 (nicked cyt *c*) exhibits the minima of ellipticity typical of helical polypeptides (*3*−*5*), it can be concluded that the N- and C-fragments upon association adopt a folded conformation characterized by a helical secondary structure similar to that of the intact protein.

In previous studies, we have emphasized that proteolysis of a protein substrate requires the binding and adaptation of the polypeptide chain at the specific stereochemistry of the protease's active site so that the flexible sites or regions in a globular protein are those more susceptible to proteolytic attack. In particular, the view was expressed that proteases do not cleave at the level of the hydrogen-bonded helical segments (*21*, *23*, *24*). Indeed, the results of the SDS−PAGE and RP-HPLC analyses of proteolytic mixtures (Figures 3 and 4) clearly show that the apoCyt−heme complex, as well as the complex of fragments 1−56 and 57−104, are rather resistant to proteolysis, due to the fact that they have acquired a helical conformation (see Figure 2). In the case of the apoCyt−heme complex, we only showed that proteolysis is significantly retarded, while with the fragment complementing system, a much detailed analysis of the rate and location of peptide bond fissions along the chain of the fragments has been carried out with the aid of SDS−PAGE and RP-HPLC analysis (Figure 4), as well as MALDI-MS (Figure 5).

Isolated Fragments 1−56 and 57−104. Fragment 57−104 is easily digested by proteinase K to small peptides. Proteolytic cleavages occur at several sites of its 48-residue chain, implying a rather unfolded and flexible state for this fragment. On the other hand, isolated fragment 1−56 is not fast degraded to many small peptides and is instead quite

resistant to proteolysis. A clear-cut interpretation of the SDS−PAGE, RP-HPLC, and MALDI-MS data (see Figures 4 and 5) is that fragment 1−56 is digested somewhat stepwise from its C-terminus, while its N-terminal portion is not attacked by the proteolytic probe. On the other hand, the far-UV CD spectrum of this fragment, even if not typical of a random coil, does not indicate a significant content of secondary structure (Figure 2B). A plausible explanation for the proteolysis data is that isolated fragment 1−56 adopts a folded and sufficiently rigid structure to prevent its extensive proteolytic degradation, especially at its N-terminus. This is consistent with the following considerations. The heme prosthetic group is covalently attached to Cys14 and Cys17 in the fragment via thioether linkages, and the imidazole group of His18, in native cyt *c*, provides the proximal heme ligand. Moreover, while in native cyt *c* Met80 coordinates the heme iron via its thioether function, in fragment 1−56 instead His26 and/or His33 can provide an additional ligand for the heme iron, besides His18. Experimental evidence of the possible involvement of His residue(s) in binding the heme in intact misligated cyt *c* has been provided (*48*−*52*). In particular, mutagenesis studies indicated that His33 appears to be the dominant sixth ligand for the heme iron in misligated bis-His cyt *c* (*50*). Indeed, in a recent study, it has been found that fragment 1−56 has two His residues axially bound to the heme iron, the native His18 and His26 or His33 (*53*). Finally, it could be well that the short N-terminal helix (residues 6−14) of the native protein is retained to some extent in isolated fragment 1−56, since the far-UV CD spectrum of the fragment provides evidence of some residual structure (see above). Therefore, the constrained structure of the fragment substrate given by the various heme ligands and the macromolecular crowding around the hydrophobic heme moiety, as well as perhaps some residual N-terminal helix, appear to hamper proteolysis in the N-terminal portion of fragment 1−56. It is interesting to observe that the proteolysis data for fragment 1−56 in isolation can be interpreted in terms of a somewhat fixed structure of the fragment substrate, even in the absence of significant secondary structure. Indeed, recent EPR measurements provided evidence that fragment 1−56 adopts a compact structure with the heme group partially shielded from solvent (*53*).

Complex of Fragments 1−56 and 57−104. Nicked cyt *c*, resulting from the association of fragments 1−56 and 57−104, is rather resistant to proteolysis, if its rate is compared with the rate of proteolytic degradation of the constituent fragments. This is explained by the fact that nicked cyt *c* is folded into a native-like secondary structure (Figure 2B) (see also the Supporting Information). The striking result of the proteolysis data is that fragment 57−104, which is degraded quickly in isolation, is instead fully resistant to proteolysis if embedded in the complex of fragments 1−56 and 57−104. If a native-like structure of nicked cyt *c* is assumed, it could be well that the C-terminal helix (residues 87−102) and the Met80 ligand for the heme iron are restored in the nicked species. Since the tertiary structure near Trp59 is mostly retained in the complex, as given by the efficient fluorescence quenching of the Trp fluorescence by the heme (see the Supporting Information), the 60's helix (residues 60−69) also likely is preserved, as well as the hydrogen bonding between the indole group of Trp59 and the propionate side chain of the heme (*54*). In native cyt *c*, the C-terminal helix of residues 87−102 strongly interacts with the N-terminal helix of residues 6−14 (see Figure 1). These structural features and interactions rigidify fragment 57−104 and make it strongly resistant to proteolytic degradation, since helices are not sites of proteolysis (*21*, *24*, *29*). On the other hand, after reaction for 1 h, mostly the chain segment from residue 49 to 56 is excised from the complex of fragments 1−56 and 57−104 (see Figure 5C). Prolonged proteolysis (up to 5 h) leads to the progressive shortening of the N-terminal component of the complex and formation mostly of fragment 1−40, while fragment 57−104 still remains uncleaved. It is interesting to observe that the chain segment which is excised from nicked cyt *c* matches that evolutionarily removed in short cyt *c* from bacteria, like that from *Pseudomonas* (*55*). This short cyt *c* is evolutionarily related to cyt *c* from eukaryotes, has the same overall folding and function, but is only 82 amino acid residues long instead of 104 (*56*−*58*). With respect to horse cyt *c*, the chain deletion in *Pseudomonas* cyt *c* occurs from residue 41 to 55. Therefore, limited proteolysis of nicked cyt *c* can be considered an in vitro protein truncation which occurred in vivo by evolution. Of interest, a semisynthetic shorter variant of horse cyt *c*, in which the *ω*-loop (*59*) comprising residues 41−55 was deleted, maintained the overall fold as well as functional properties of cyt *c* (*60*, *61*). Also, a functioning noncovalent complex of two cyt *c* fragments with deletion of the 39−58 eicosapeptide was described (*62*).

Implications for Protein Folding Studies. Among the previously studied fragment complementing systems of cyt *c* (*35*, *36*, *42*, *43*), the present complex of fragments of 1−56 and 57−104 offers the unique feature of being formed exactly by the two protein fragments encoded by the two exons (coding sequences) of cyt *c*. In fact, the intron−exon splice junction in the gene of this protein occurs at Gly56 (*39*). It has been suggested that introns mark the location of subdomains and domains in a protein and that the corresponding protein fragments may retain some independent structure and function (*40*, *41*, *63*, *64*). Moreover, it appears that the intron−exon junctions map at protein surfaces (*41*) and identify flexible sites in a protein (*40*). Isolated fragments 1−56 and 57−104 do not exhibit independent folding to a native-like secondary structure, as given by far-UV CD measurements (see Figure 2B). Nevertheless, their not fully random structure likely permits adoption of a "native format" sufficient to dictate intermolecular recognition between the two fragments, thus shifting the equilibrium toward the native state of the fragments and thus formation of a native-like nicked cyt *c*.

Following the classical study on bovine pancreatic ribonuclease (*65*), there have been numerous reports indicating that globular proteins can tolerate the proteolytic fission of a single peptide bond along their polypeptide chain without impairing stability and often biological activity. Moreover, in numerous cases it has been possible to isolate the individual fragments and then to reassemble them into a noncovalent, stable protein complex (nicked protein). Thus, it is perhaps surprising that a change in chain connectivity does not alter the structure and stability of a localized element of structure, thereby favoring overall alternative conformations of the protein. The straight explanation for this is that the specific folding of that protein can tolerate a change in

its covalent structure at a specific site and that there are other interactions and forces which are sufficiently strong to keep the folded state of the protein nearly unchanged. Taniuchi and co-workers, on the basis of numerous experimental studies on fragmentation and complementation on staphylococcal nuclease and horse cytochrome *c* (*42*, *43*), developed the concept of a "permissible site" for cleaving a protein without much altering its three-dimensional structure. These earlier studies have indicated that the permissible regions for cleavage without main disruption of the ordered structure of the protein are usually located in exposed and flexible loops of the protein molecule. On this basis, it is understood why the nicked cyt *c* investigated herewith is efficiently formed by complementation of fragments 1−56 and 57−104, since the chain discontinuity occurs at the level of an irregular loop (see Figure 1), characterized by exposure and flexibility. The conformational mobility of the Gly56−Ile57 peptide bond in intact cyt *c* has been inferred from NMR studies on the methyl resonance of Ile57 (*66*). Indeed, it has been amply demonstrated by protein engineering experiments that globular proteins can tolerate extensive side chain replacements and changes at their exposed and flexible regions, while the most detrimental mutations for structure and stability are those occurring at buried sites of the protein (*67*−*69*).

In recent years, we have seen a strong revival of the use of fragment complementing systems (*70*−*73*), in view of their general utility in protein science and, in particular, as a means of investigating protein folding. In fact, the overall mechanisms of protein folding of a single chain and a complementing fragment system likely are similar, the difference being that the mechanism is intramolecular and intermolecular, respectively (*74*). When studied in isolation, the fragments can provide information about the early events in protein folding, since they cannot experience the specific, long-range interactions of the parent, native protein (*75*). Complementing protein fragments, therefore, can be used to dissect the difficult problem of protein folding, allowing the study of steps in this complex phenomenon still awaiting description in molecular, kinetic, and thermodynamic terms (*76*, *77*). Moreover, as already emphasized by Taniuchi and co-workers (*43*), fragment complementation allows the study of folding and unfolding aspects of proteins under physiological conditions, without requiring the use of extreme solvent conditions (acid or alkaline pH, denaturants, high temperature). Therefore, the cyt *c* fragment system described herewith appears to be highly interesting and useful for protein folding studies.

Conclusion. The use of proteolytic enzymes for probing protein conformation has been highlighted previously (*21*−*27*, *29*), but the merit of this study is that it shows how detailed and clear-cut protein structural and dynamic informations can be obtained by limited proteolysis experiments. This is a simple biochemical technique, which can exploit the recent advances in MS methodologies for protein fragment identification and, therefore, requires very minute amounts of protein sample. Here, we made use of the cyt *c* system as a model protein, which shows the advantages of being extremely well-known in terms of its three-dimensional structure (*54*, *78*), mechanism of folding (*49*, *79*−*83*), and function (*58*). Some aspects of structure and stability of cyt *c* have been investigated previously using proteolysis experiments by Taniuchi and co-workers (*42*, *43*) and others (*84*−*86*). Overall, the results of this study show that protein folding leads to enhanced resistance to proteolysis, while local dynamics or segmental mobility of the polypeptide chain dictates the sites or regions prone to preferential or selective proteolysis. Proteolysis data can be interpreted with confidence in terms of structure and dynamics of the protein substrate, and thus, the use of proteolytic probes should be viewed as an approach that is complementary to those involving the most common and classical spectroscopic techniques.

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SUPPORTING INFORMATION AVAILABLE

CD spectra of cyt *c* and the complex of fragments 1−56 and 57−104 in the near-UV and Soret regions; fluorescence emission spectra upon excitation at 290 nm of cyt *c*, fragment 1−56, fragment 57−104, and the complex of fragments 1−56 and 57−104; absorbance in the visible and near-IR regions of the complex of fragments 1−56 and 57−104 and intact cyt *c*; and gel filtration chromatograms of cyt *c*, fragment 1−56, fragment 57−104, and the complex of fragments 1−56 and 57−104. This material is available free of charge via the Internet at http://pubs.acs.org.

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