Folding of Yeast Iso-1-AM Cytochrome *c*†

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ABSTRACT: We describe a specific modification of iso-1 cytochrome *c* which results in blocking a single free sulfhydryl group. The derivative differs from the unmodified protein by the introduction of a small, uncharged group, thus maintaining the same charge balance as the native protein. The modified protein, obtained by treatment of iso-1 cytochrome *c* with iodoacetamide, has an activity indistinguishable from that of the unmodified protein in the lactate dehydrogenase–cytochrome *c* reductase system from yeast and has the same stability toward denaturation by guanidine hydrochloride. The kinetics of fluorescence changes associated with the guanidine hydrochloride induced folding–unfolding transition for modified iso-1 cytochrome *c* (iso-1-AM) have been investigated throughout the transition zone by using stopped-flow mixing. The results are compared to those for the yeast isozyme, iso-2 cytochrome *c*. The main features of the fluorescence-detected folding kinetics are similar, as might be expected for homologous proteins; however, the limiting value of the fraction of fast refolding protein (α_2) below the transition zone is smaller for iso-1-AM (~0.7) than for iso-2 (~0.9).

Few monomeric proteins are better suited for studies of how amino acid sequence relates to protein structure and function than cytochrome *c*. Homologous proteins have been isolated from a wide variety of sources, and sequencing studies have left us with a greater wealth of primary structural data than for any other class of small monomeric proteins (Dickerson & Timkovich, 1975). Tertiary structures for representative members of different subclasses of the cytochrome *c* family have been determined and comparison shows that all are folded in a similar manner but differ by additions or deletions of amino acids in regions which connect one helical segment of the protein to another (Dickerson, 1980).

Comparison of homologous proteins can be a productive approach to understanding protein structure and function, but it must be kept in mind that crucial relationships between structure and function will be conserved through evolution. On the other hand, mutations resulting in altered proteins are less subject to selective pressures. Therefore, a comparison of mutant and wild-type cytochrome *c*'s can be expected to yield new information complementary to that obtained from studies of homologous proteins.

Iso-1 cytochrome *c* from yeast is one of the best systems for the study of mutant cytochrome *c*'s. The iso-1 cytochrome *c* gene from the yeast *Saccharomyces cerevisiae* is the most thoroughly characterized of all cytochrome *c* genetic systems. A large number of structural mutants have been obtained and the primary structures of many of the resulting altered proteins characterized (Sherman & Stewart, 1978). Thus iso-1 cytochrome *c* from yeast is the system of choice for studies of how mutations resulting in changes in amino acid sequence are expressed in terms of alterations in tertiary structure formation and in functional properties.

Unfortunately, some studies of iso-1 cytochrome *c* have been hindered by the presence of a single free sulfhydryl group near the carboxy-terminal end of the protein. On oxidation, this leads to the formation of variable amounts of disulfide-linked dimers which can be disrupted only by returning the protein to reducing conditions. This makes studies of the Fe(III) form of the monomeric protein difficult since reduction of the disulfide bonds by thiol reagents brings about reduction of the heme. Also, thiol reagents can be expected to compete with the normal heme ligands (methionine and histidine) and thus alter the properties of the protein when in the presence of such reagents.

In order to make iso-1 cytochrome *c* and mutants of iso-1 more amenable to detailed physical and enzymatic studies, we have determined procedures by which the free sulfhydryl group can be specifically blocked by reaction with iodoacetamide. The modified protein (iso-1-AM)[1] should have the same overall charge as the native protein and behaves in the same manner as the unmodified protein in a variety of spectroscopic and enzymatic tests.

Our overall goal is to use studies of mutant and homologous cytochrome *c*'s to investigate how changes in amino acid sequence affect protein folding reactions. The kinetic and equilibrium properties of the guanidine hydrochloride induced folding and and unfolding of iso-1-AM are reported here. Fluorescence has been used to monitor the kinetics of folding of iso-1-AM due to its great sensitivity and to the plausibility of interpreting cytochrome *c* fluorescence in terms of changes in the heme to tryptophan distance (Tsong, 1974). The measurable kinetic parameters, the relative amplitudes, and time constants associated with the observed kinetic phases are compared to those for the yeast isozyme, iso-2 cytochrome *c* (Nall & Landers, 1981; Nall, 1983). Iso-2 contains four additional amino acids at the amino terminus as well as 17 differences in sequence at homologous positions. These differences in sequence are conservative (hydrophobic for hydrophobic, polar for polar, etc.) or occur on the surface of the protein.

The results presented here support the idea that proteins with different primary structures but homologous tertiary structures have similar folding kinetics. This may indicate a common folding pathway. Nevertheless, a difference in the fraction of fast refolding material (α_2) for refolding experi-

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[1] Abbreviations: TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; iso-1, iso-1 cytochrome *c* from *Saccharomyces cerevisiae*; iso-1-AM, iso-1 specifically blocked at cysteine-102 by reaction with iodoacetamide; $NaDodSO_4$, sodium dodecyl sulfate; τ, reciprocal of the apparent reaction rate constant.

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ments ending below the unfolding transition zone is observed.

Materials and Methods

Protein and Reagents. Trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK)[1] was from Worthington. Iso-1 cytochrome *c* from *Saccharomyces cerevisiae* (type VIII) and yeast lactate dehydrogenase (cytochrome b_2, ferricytochrome *c* oxidoreductase, EC 1.1.2.3 type IV S) were obtained from Sigma Chemical Co. and used without further purification. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of iso-1 cytochrome *c* (Laemmli, 1970) indicated a homogeneous polypeptide of about 12 000 g/mol. Iodoacetamide, dithiothreitol (DTT), and L(+)-lactic acid were also from Sigma. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was from Aldrich. Iodo[1-^{14}C]acetamide at a specific activity of 53 Ci/mol was obtained from Amersham and diluted by 50-fold with cold iodoacetamide. Radiochemical purity was checked by reaction with an excess of glutathione (Sigma) followed by electrophoresis on 10 × 10 cm cellulose thin-layer chromatography plates. Unreacted and carboxymethylated glutathiones were located by spraying with ninhydrin (Pierce Chemical) followed by autoradiography (see Peptide Mapping and Autoradiography). From the resulting autoradiogram, it is estimated that better than 98% of the ^{14}C-labeled reagent had reacted with glutathione. Resins for cation-exchange chromatography were from Bio-Rad while Sephadex for gel filtration was from Sigma. Guanidine hydrochloride (Gdn·HCl) was from Heico and was used without further purification.

Chemical Modification and Purification of Carboxymethylated Iso-1 Cytochrome c. One hundred milligrams of iso-1 cytochrome *c* was dissolved in 4 mL of 0.05 M EPPS [4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid], pH 8.7. Thirty microliters of 0.5 M dithiothreitol (DTT) was added to the solution to disrupt any disulfide dimers. The solution was incubated at room temperature for 15 min and then passed over a 3 × 50 cm (260 mL bed volume) Sephadex G-25-300 column equilibrated with 0.05 M EPPS at 4 °C to remove the DTT. The protein concentration was determined by measuring the absorbance at 410 nm [the molar extinction coefficient of horse cytochrome *c*, $\epsilon = 106 \times 10^3$ L/(mol·cm) (Margoliash & Frohwirt, 1959), was used to estimate the concentration]. The concentration was usually a little higher than 10^{-4} mol/L. The additional volume of liquid needed to bring the protein concentration down to 10^{-4} mol/L was calculated and an iodoacetamide solution containing 0.3 mg of iodoacetamide/mg of protein prepared in this volume of deionized water. The protein solution and iodoacetamide solution were mixed (total volume was 80 mL) and incubated at room temperature in the dark while stirring under a stream of nitrogen. After 1 h, an aliquot was removed from the reaction mixture and passed over a Sephadex G-25-300 column equilibrated with 0.1 M sodium phosphate buffer, pH 8.0, to remove reagent. A free sulfhydryl assay (see Assays) was performed immediately to determine the progress of the reaction. Additional aliquots were removed and free sulfhydryl assays performed until the protein was 70–80% blocked. The reaction was stopped by passing the solution over a 5 × 100 cm (1.6 L bed volume) Sephadex G-25-300 column equilibrated with 0.05 M sodium phosphate buffer, pH 7.2.

For separation of modified from unmodified protein, cation-exchange column chromatography was performed, essentially as described by Schweingruber et al. (1978). The protein solution was loaded onto a 2.5 × 40 cm (170 mL bed volume) Bio-Rex 70 (200–400 mesh) column equilibrated with 0.1 M sodium phosphate buffer, pH 7.2 at room temperature. The column was washed with 3 column volumes of buffer to which DTT had been added to a concentration of 10^{-3} mol/L. Two 500-mL volumes of buffer (0.1 M sodium phosphate, pH 7.2, 10^{-3} mol/L DTT), one of which was 0.8 M in sodium chloride, were used to elute the protein with a 0–0.8 M sodium chloride gradient.

Assays. The free sulfhydryl concentration of a solution of iso-1 cytochrome *c* was determined by using the reagent of Ellman (1959). The method relies on the spectrophotometric detection of thionitrobenzoate anion released when DTNB reacts with free sulfhydryls. For the present application there are two problems. First, the wavelength usually used to monitor the reaction, 412 nm, is near an absorbance maximum for a heme absorbance band. Second, oxidized heme is reduced on reacting with DTNB, giving spectral changes which interfere with those due to thionitrobenzoate anion. Accordingly, we have confined our free sulfhydryl assays to the fully reduced form of iso-1 cytochrome *c* and have used a wavelength of 434 nm to monitor the reaction. This wavelength is an isosbestic point for spectral changes of the protein due to changes in oxidation state, has a lower molar extinction for the protein, and has an extinction coefficient of 1.11×10^3 L/(mol·cm) for thionitrobenzoate anion. The extinction coefficient for the thionitrobenzoate anion was determined from the known extinction at 412 nm [13 600 L/(mol·cm); Habeeb, 1972], and the ratio of the absorbances at 412 and 434 nm of a DTNB solution fully reacted with β-mercaptoethanol. For reduction of the heme iron atom and disruption of any disulfide-dimerized protein, a 2-fold molar excess of DTT was added to the protein solution. The reaction was allowed to proceed for 10 min at room temperature, after which the solution was passed over a Sephadex G-25-300 column equilibrated with buffer [0.1 M sodium phosphate, pH 8.0, and 10^{-3} M EDTA (ethylenediaminetetraacetic acid)]. Free sulfhydryl assays were performed as described by Habeeb (1972) immediately following elution of the protein from the Sephadex column.

Activity of native and modified iso-1 cytochrome *c* was compared by using the yeast lactate dehydrogenase assay as described by Parr et al. (1978) except that the reaction was initiated by the addition of 10 μL of lactate dehydrogenase (1.0 unit/mL) and carried out in 0.1 M sodium phosphate buffer, pH 7.2, at 20 °C. Native protein used in the assay was shown to be better than 90% monomer by gel filtration on a 0.7 × 18 cm Sephadex G-50-40 column equilibrated with 0.1 M sodium phosphate, pH 7.2.

Peptide Mapping and Autoradiography. Digestion by trypsin was performed by dissolving 5 mg of iso-1 cytochrome *c* in 0.75 mL of 0.032 M NH_4HCO_3, pH 7.8, adding 0.1 mL of trypsin stock solution (5 mg/mL in 0.06 M HCl), and incubating for 2.75 h at 37 °C. The digest was lyophilized, resuspended in 2.5 mL of water and lyophilized (twice), and resuspended in the paper chromatography buffer or thin-layer electrophoresis buffer. Peptide mapping on paper was by the method of Titani et al. (1964) except that 50 × 60 cm paper was used with chromatography along the 60-cm dimension and electrophoresis at 2500 V for 45 min along the 50-cm dimension. Peptide mapping was also performed on cellulose thin-layer plates (10 × 10 cm, E.M. Laboratories 5611-9H) by a method similar to that described by Gautsch et al. (1978). Peptides were located on both paper and thin-layer peptide maps by spraying with fluorescamine (Felix & Jimenez, 1974). Autoradiography of tryptic maps of iso-1 cytochrome *c* modified with iodo[^{14}C]acetamide was for 1–4 weeks using 3 M Trimax 8 X-ray film.

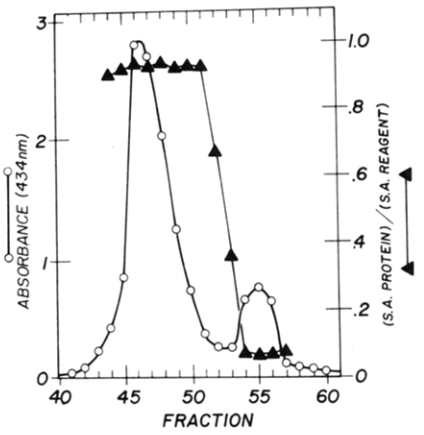

FIGURE 1: Separation of the modified protein, iso-1-AM, from native iso-1 by column chromatography. The protein, partially reacted with iodo[^{14}C]acetamide, is bound to the cation-exchange resin (Bio-Rex 70, 200–400 mesh) in 0.1 M sodium phosphate buffer, pH 7.2, and eluted with a 0–0.8 M sodium chloride gradient. The absorbance at 434 nm (○) and the ratio of the specific activity of the protein to the specific activity of the input label (▲) are shown. The second peak to elute (fraction 55) reacts with DTNB, indicating the presence of free sulfhydryls. The first peak (fraction 46) does not react with DTNB.

Amino Acid Analysis. Proteins to be analyzed were hydrolyzed for 24 h in 6 N HCl in vacuo. Amino acid composition was determined on a Durrum D-400 amino acid analyzer.

Spectrophotometric Measurements. Absorbance spectra were obtained on a Hewlett-Packard UV–visible spectrophotometer (Model 8450A) in 0.1 M sodium phosphate buffer, pH 7.2 at 20 °C. Fluorescence measurements were made on an Aminco–Bowman spectrophotofluorometer at 20 °C. The excitation wavelength was 280 nm, and fluorescence was detected at 340 nm by using entrance and exit slits of 3 mm.

Kinetic Measurements. Kinetic measurements were made on a Durrum D-110 flow system in combination with a lamp housing, grating monochromator and quartz focusing optics (Oriel Corp.). Fluorescence excitation was at 287 nm by using a Hg–Xe 200-W arc lamp (Canrad-Hanovia). Fluorescence was detected by mounting a photomultiplier tube (EMI 9558 QB) at right angles to the optical path. A band-pass interference filter centered at 350 nm was used to eliminate stay excitation light. The split-time base mode of a Biomation transient recorder was used to store both fast and slow phase kinetic data from single mixing experiments, thus increasing the accuracy of relative amplitude measurements.

An interactive computer program was used to determine the time constants and relative amplitudes by direct comparison of the digitized kinetic traces to a trial function of the form $\Delta A(t) = A + B \exp[-(t/\tau_1)] + C \exp[-(t/\tau_2)]$. Additional procedures relating to instrumentation and data analysis have been described previously (Nall & Landers, 1981; Nall, 1983).

Results

Reaction with Iodoacetamide Blocks the Free Sulfhydryl. Reaction of iso-1 cytochrome *c* with iodoacetamide leads to protein which is unable to react with a free sulfhydryl reagent, DTNB. In principle, this could be due to blockage of the sulfhydryl by chemical modification, or to the formation of disulfide bonded dimers. Since the free sulfhydryl assay is preceded by treatment of the protein with DTT, a reagent known to disrupt disulfide bonded dimers (see Figure 5), the best explanation for the lack of reaction with DTNB is that the iodoacetamide has chemically blocked the free sulfhydryl.

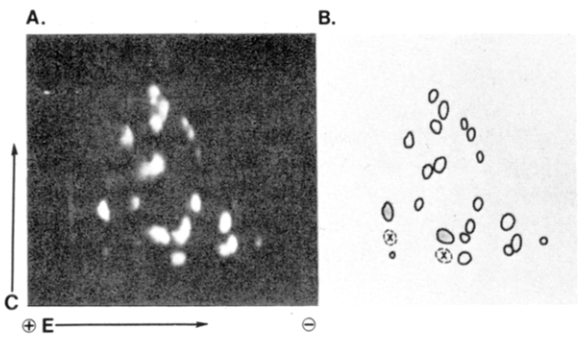

FIGURE 2: (A) A tryptic peptide map of ^{14}C-labeled iso-1-AM by the method of Titani et al. (1964). In (B) the resulting autoradiogram has been superimposed on an outline of the tryptic map. Tryptic maps of native iso-1 give an identical pattern except that the labeled spots are absent and two additional spots appear at the indicated positions (×).

Ion-exchange chromatography of the protein after reaction with iodoacetamide is shown in Figure 1. The DTNB reactive material chromatographs at the position of native iso-1, while the DTNB negative material elutes a little sooner. We conclude that iso-1-AM is separable from iso-1 by column chromatography. This is confirmed by the elution pattern observed for labeled and unlabeled protein when iodo[^{14}C]-acetamide is used as the modifying reagent (Figure 1) and by the fact that reaction for longer times leads to an increase in the fraction of material in the DTNB negative peak (not shown).

One Mole of Reagent Is Incorporated per Mole of Protein. In Figure 1, it is seen that the ratio of the specific radioactivity of the protein to that of the reagent is close to one for the peak fractions corresponding to the modified protein. This demonstrates that 1 mol of iodoacetamide reacts with 1 mol of protein. Since the modified material does not react with DTNB, we are left with three possibilities: (1) iodoacetamide reacts with the free sulfhydryl blocking it directly, (2) iodoacetamide reacts with a nearby amino acid and shields the sulfhydryl from reaction with DTNB, and (3) reaction with an amino acid residue in the protein induces a conformational change which buries the sulfhydryl, thus protecting it from reaction with DTNB. The known chemical specificity of iodoacetamide for free sulfhydryls under the conditions used for the modification reaction makes the first possibility the most likely.

Peptide Mapping and Autoradiography. Two different systems for peptide mapping were used with essentially the same results. Mapping of tryptic digests of the modified and unmodified protein showed that two peptides from the modified protein had altered positions. A representative peptide map of modified iso-1 cytochrome *c* using the paper mapping system of Titani et al. (1964) is shown in Figure 2A. The resulting autoradiogram is shown in Figure 2B. A thin-layer method similar to that of Gautsch et al. (1978) showed about the same number of resolved tryptic peptides and two with altered positions relative to the unmodified protein (data not shown). In both cases autoradiography confirmed that the two peptides with altered positions were those which had incorporated ^{14}C label. Heavily overexposed autoradiograms of tryptic maps in both systems gave no indication of ^{14}C incorporation into any other peptides.

The amino acid compositions of the peptides separated by tryptic mapping have been determined by Titani et al. (1964). Comparison of our peptide maps to those of Titani et al. (1964) indicates that the peptides in iso-1 with altered positions in iso-1-AM both are from the carboxy-terminal end of the

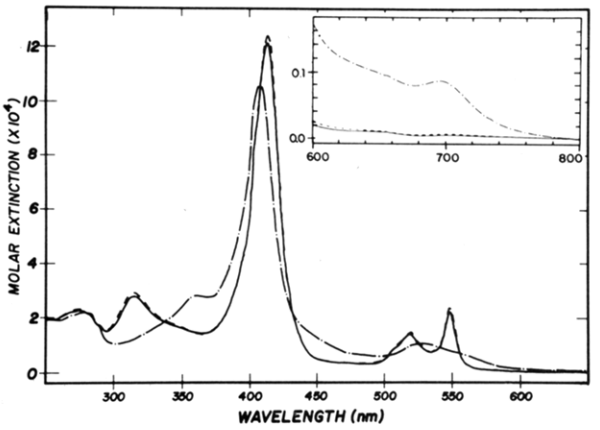

FIGURE 3: Absorbance spectra of iso-1-AM. Spectra of the oxidized (-·-) and reduced (—) forms are shown. After carbon monoxide is bubbled through a solution of the reduced protein, there is little change (---). The insert shows the 695-nm absorbance band which is indicative of the functional, native form of cytochrome *c* in which methionine and histidine side chains are axial ligands of the heme (Schechter & Saludjian, 1967; Ridge et al., 1981).

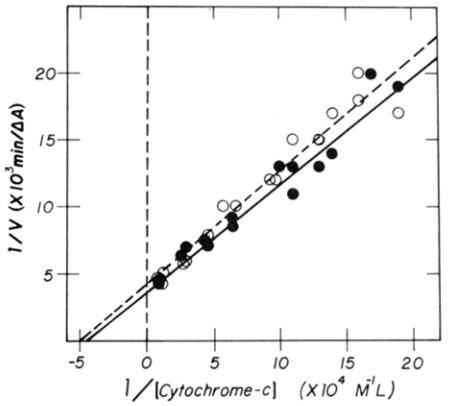

FIGURE 4: Double-reciprocal plot of the rate of reduction of iso-1-AM (●) and native iso-1 (○) in a yeast lactate dehydrogenase–cytochrome *c* reductase assay. The lines are least-squares fits for the iso-1 data (---) and the iso-1-AM data (—).

protein and contain the free sulfhydral residue of the native protein. Two carboxy-terminal peptides (Lys-Ala-Cys-Glu and Ala-Cys-Glu) are produced by tryptic digestion due to the presence of Lys-Lys in the protein sequence. One of the peptides arises from cleavage between the Lys residues and the other from cleavage after both lysines.

Amino Acid Analysis. The amino acid composition of total hydrolysates of the modified protein gives 1.1 mol of (carboxymethyl)cysteine/mol of protein. This result taken together with the peptide mapping results shows that iodoacetamide reacts specifically at cysteine, blocking the free sulfhydryl group.

Comparison of the Properties of the Modified and the Native Protein. (1) Spectral Properties Are Identical. Comparison of spectra of the fully oxidized and the fully reduced proteins at all wavelengths between 250 and 800 nm shows that the spectra of native iso-1 and iso-1-AM are identical within experimental error (a few percent in total absorbance). In Figure 3, spectra of the fully oxidized and the fully reduced forms of the modified protein are presented. They exhibit all of the characteristic features of cytochrome *c* absorbance spectra. Furthermore, bubbling carbon monoxide through a solution of reduced iso-1-AM gives no significant spectral change, indicating a well-protected heme environment.

(2) *Enzymatic Properties Are the Same.* The ability of iso-1-AM to function as an efficient electron acceptor has been

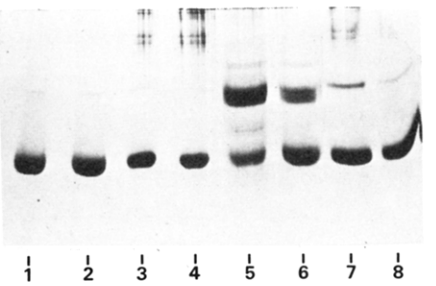

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of iso-1-AM and native iso-1 cytochrome *c*. The method is that of Laemmli (1970). From left to right, the samples are (1) iso-1-AM, (2) iso-1-AM boiled before loading, (3) iso-1-AM loaded with DTT in the sample buffer, (4) iso-1-AM boiled with DTT before loading, (5) iso-1, (6) iso-1 boiled, (7) iso-1 with DTT in the sample buffer, and (8) iso-1 boiled with DTT.

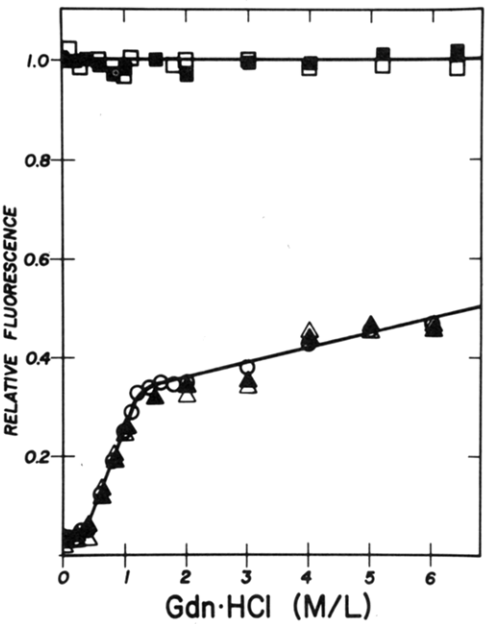

FIGURE 6: Gdn·HCl-induced equilibrium unfolding transitions of iso-1 and of iso-1-AM as monitored by relative fluorescence. The excitation wavelength is 280 nm, and fluorescence intensity is monitored at 340 nm. Protein concentration is 5×10^{-6} M in 0.05 M Hepes, pH 7.0 at 20 °C. The relative fluorescence of iso-1-AM (○), iso-1 (△, ▲), and free tryptophan (□, ■) are indicated. The filled triangles indicate protein samples which were unfolded by exposure to high Gdn·HCl concentrations and then refolded by diluting out the Gdn·HCl. The open and filled squares are tryptophan fluorescence for two separate experiments.

tested in the yeast lactate dehydrogenase–cytochrome *c* reductase system. As shown in Figure 4, the kinetic parameters of iso-1-AM are essentially indistinguishable from those of native iso-1 cytochrome *c*.

We have also compared the properties of iso-1 and disulfide dimers of iso-1 in this assay. When the data are plotted as in Figure 4 where the horizontal axis is expressed in (mol of heme)$^{-1}$, then the apparent K_m for the dimer is about twice that of the monomer. The V_{max} of the dimer was not significantly different from that of the monomers.

(3) *Iso-1-AM Does Not Form Disulfide Dimers.* In Figure 5, $NaDodSO_4$–polyacrylamide gel electrophoresis patterns are compared for native iso-1 and iso-1-AM. Native iso-1 and iso-1-AM have been loaded on the gel with and without DTT, and with and without boiling in $NaDodSO_4$. The iso-1 samples loaded without DTT show a significant amount of dimeric material while samples of iso-1-AM show only insignificant amounts of covalent dimer formation (<1%). This demon-

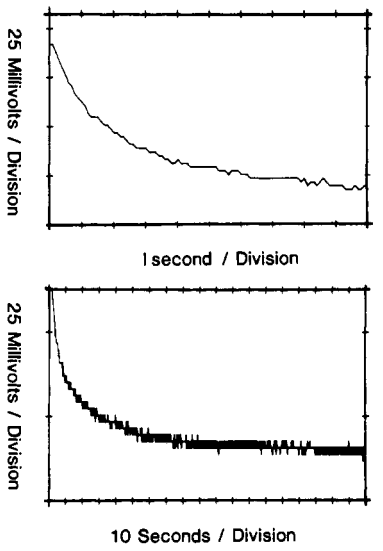

FIGURE 7: Kinetics of fluorescence changes on refolding of iso-1-AM following a Gdn·HCl concentration jump from 1.5 to 0.3 M. Two kinetic phases are detected in the stopped-flow time range, (top panel) a fast phase τ_2 and (bottom panel) a slow phase τ_1. All solutions contain 0.1 M sodium phosphate buffer, pH 7.2. Temperature is 20 °C.

strates that iso-1 readily forms covalent dimers and that blocking the free sulfhydryl by reaction with iodoacetamide eliminates covalent dimer formation.

(*4*) *Guanidine Hydrochloride Unfolding.* In Figure 6, relative fluorescence measurements of iso-1 and of iso-1-AM on unfolding in Gdn·HCl are shown. The fact that the unfolding transitions for iso-1 and iso-1-AM are superimposable indicates that the chemical modification has no effect on the overall thermodynamic stability of iso-1-AM. Since the transition curve for protein that has been previously unfolded is the same as that of native protein, the unfolding transition is a reversible process.

Kinetics of Folding. In Figure 7 typical kinetic traces are presented for refolding which starts slightly above the equilibrium Gdn·HCl-induced transition zone and which ends under conditions where the protein is close to completely folded. The usual pattern of fast and slow kinetic phases observed for a variety of small globular proteins is observed (Kim & Baldwin, 1981). Moreover, for refolding experiments ending below the transition zone, the limiting value of the relative amplitude for the fast phase (α_2) is close to that observed for homologous cytochrome *c*'s (Ikai et al., 1973; Tsong, 1976).

In Figure 8 folding and unfolding data are combined and the relative amplitudes and time constants plotted vs. the final Gdn·HCl concentration. The resulting behavior of these kinetic parameters is seen to be qualitatively similar to that observed for homologous proteins (Ikai et al., 1973; Tsong, 1976). In particular the time constants are in similar time ranges, and the faster phase (τ_2) has an inflection at the Gdn·HCl concentration corresponding to the midpoint of the equilibrium transition curve. The sigmoidal behavior of α_2, rising from about 0.7 near the lower edge of the transition to 1.0 above the transition zone, is typical of the behavior predicted by a simple three-state kinetic mechanism (Hagerman & Baldwin, 1976; Hagerman, 1977).

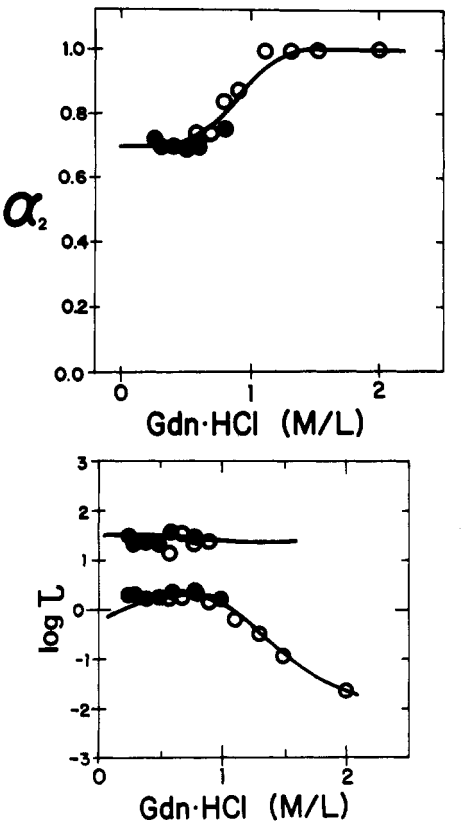

FIGURE 8: Iso-1-AM. The Gdn·HCl dependence of (top panel) the relative amplitude, α_2, of the fast reaction, and (bottom panel) of the time constants associated with the fluorescence detected phases in folding (●) and unfolding (○). Assuming the Gdn·HCl dependence of the time constants indicated in the bottom panel (—), the relative amplitude behavior shown in the top panel (—) is predicted on the basis of a three-state model (Hagerman & Baldwin, 1976; Hagerman, 1977). The initial Gdn·HCl concentration was 1.5 M for refolding and 0.3 M for unfolding experiments. The final Gdn·HCl concentration is indicated on the *x* axis. Other conditions are as described in Figure 7.

Discussion

Iodoacetamide Reacts Specifically with Iso-1 Cytochrome c. There are several lines of evidence which make this conclusion inescapable. First, the stoichiometric incorporation of label indicates that a single residue has been modified. Second, the presence of stoichiometric amounts of (carboxymethyl)cysteine in the amino acid analysis of total hydrolysates shows that the residue modified is a cysteine. Third, tryptic peptide mapping and autoradiography rule out the possibility of reaction at other sites being reversed during the hydrolysis preceding amino acid analysis. Finally, the modified peptides are from the region of the native protein which contains the free sulfhydryl group.

Iso-1-AM and Monomeric Native Iso-1 Have Similar Properties. Our limited comparison of the functional and physical properties of native iso-1 cytochrome *c* and iso-1-AM indicates that these two proteins behave in an identical manner as long as the native protein is monomeric. This result is expected since the crystal structure of cytochrome *c* from tuna and a comparison of the amino acid sequence of cytochrome *c*'s from 60 eukaryotic species indicate that the modified residue is on a hypervariable region of the molecular surface with little or no function (Dickerson & Timkovich, 1975). Moreover, being a single residue in from the carboxy-terminal end of the protein, it is likely that this region of the polypeptide chain is involved in a great deal of motion in both the native and modified proteins. If so, this would tend to minimize any effects of chemical modification on more ordered segments of the polypeptide chain. Support for this view comes from the electron paramagnetic resonance studies of Drott et al. (1970). A bromoacetamide derivative of 2,2,5,5-tetra-

methylpyrrolidinyl-1-oxy was used to spin-label iso-1 cytochrome *c* at what was probably cysteine-102. Electron paramagnetic resonance spectra of this derivative showed weak immobilization of the label, suggesting that the spin-label could reorient freely relative to the protein.

Iso-1-AM Is Suitable for Physical and Enzymatic Studies under Conditions Where Iso-1 Is Not. High pH, high temperature, and high protein concentrations are all conditions which favor the formation of intermolecular disulfide dimers. Since a variety of functional and physical studies require these conditions, iso-1-AM is superior to native iso-1 for such investigations. For most proteins which contain free sulfhydryls, the addition of sulfhydryl reagents to the buffers is a convenient way to avoid intermolecular disulfide bonding. However, these reagents interfere with the optical properties of the solutions in the ultraviolet region and thus complicate the application of spectroscopic techniques. In the present case a more serious problem with the use of sulfhydryl reagents is that at the concentrations necessary to inhibit dimer formation, they reduce the heme iron. Thus, physical studies of the oxidized protein, and functional studies involving oxidation or reduction of the heme iron, are impossible in the presence of these reagents. As we have shown, iso-1-AM may be used to study the properties of the monomeric protein uncontaminated by covalent disulfide dimers in the absence of disulfide reagents.

Free Energy Differences between Iso-1-AM, Iso-2, and Horse Cytochrome c Are Consistent with Differences in Sequence. Even though the existence of multiple kinetic phases in folding is firmly established, a two-state treatment of equilibrium unfolding transitions is justified on empirical grounds (Lapanje, 1978). Two-state behavior at equilibrium and multistate kinetic behavior are compatible with one another as long as intermediate species (1) are present in undetectably low concentrations at equilibrium and (2) are significantly populated only transiently during a kinetic experiment. Thus a two-state treatment has been used to obtain apparent equilibrium constants and unfolding free energies ($\Delta G°_u$) for iso-1-AM from data presented in Figure 6. In order to obtain $\Delta G°_u$ (ΔG_u in the absence of denaturant) for iso-1-AM a simple extrapolation procedure suggested by Schellman (1978) has been used in a manner previously described for iso-2 cytochrome *c* (Nall & Landers, 1981). The result is given in Table I where $\Delta G°_u$ for iso-1-AM is compared to $\Delta G°_u$ for iso-2 (Nall & Landers, 1981) and horse cytochrome *c* (Knapp & Pace, 1974; McLendon & Smith, 1978).

While our understanding of the thermodynamic stability of proteins is based on estimates of the relative contributions of differences in chain entropy, hydrogen bonding, and hydrophobic interactions between the folded and unfolded states, quantitative models (Tanford, 1962; Brandts, 1964) have not been particularly successful in rationalizing observed differences in the stabilities between nonhomologous proteins. The discrepancy is usually ascribed primarily to the fact that the free energy of stabilization of a protein is a small difference between very large factors of opposite signs. Since the magnitudes of the free energies of stabilization of proteins are comparable to (if not smaller than) the estimated errors in model compound data used to evaluate the contributions to stability, the predictive value of such models is lost. In principle improved model compound data, especially transfer free energies of model compound amino acids and peptides, from solvents which simulate the protein interior (e.g., ethanol) to water might help. In practice, the uniqueness of the environments of each amino acid in proteins makes it impossible to find a universal solvent which adequately simulates the interior of a protein for all amino acids, or even a single amino acid in different proteins. For a series of homologous proteins this problem is partially obviated since predicted differences in stability are based on differences in amino acids which are found in homologous environments. Thus, if our basic understanding of the factors which contribute to protein stability is correct, improved agreement between observed and calculated stabilization free energies is expected for a homologous series.

Table I: Comparison of Experimental and Predicted Differences in $\Delta G°_u$

protein	exptl $\Delta G°_u$ (kcal/mol)[a]	exptl $\Delta\Delta G°_u$ (kcal/mol)[b]	calcd $\Delta\Delta G°_u$ (kcal/mol)[c]
horse cytochrome *c*	7.3[d]	(0)	(0)
iso-1-AM cytochrome *c*	2.1[e]	−5.2	−3.9
iso-2 cytochrome *c*	3.1[f]	−4.2	−3.3

[a] Experimental free energy of unfolding using a linear extrapolation of the two-state free energy to 0 M Gdn·HCl [a method suggested by Schellman (1978)]. The procedure used to obtain these values from equilibrium Gdn·HCl unfolding curves has been described (Nall & Landers, 1981). [b] Difference in the experimental $\Delta G°_u$ for a given protein and that of horse cytochrome *c* [$\Delta\Delta G°_u = \Delta G°_u - \Delta G°_u$ (horse)]. [c] Expected difference in $\Delta G°_u$ relative to $\Delta G°_u$ for horse cytochrome *c* estimated from transfer free energies of buried side chains (Kawaguchi & Noda, 1977; Nall & Landers, 1981). [d] Experimental $\Delta G°_u$ for horse cytochrome *c* from Knapp & Pace (1974) (7.17 kcal/mol) and McLendon & Smith (1978) (7.38 kcal/mol) measured at 25 °C. [e] Experimental $\Delta G°_u$ for iso-1-AM cytochrome *c* at 20 °C, calculated from data given in Figure 6. [f] Experimental $\Delta G°_u$ for iso-2 cytochrome *c* from Nall & Landers (1981).

In Table I, column one, stabilization free energies ($\Delta G°_u$) are given for cytochrome *c* from horse and for iso-1-AM and iso-2 from yeast (*Saccharomyces cerevisiae*). In column two the observed differences in stability between the proteins from yeast and horse cytochrome *c* are given while in column three predicted differences in stability are listed. The calculated values are arrived at by using predictive models of protein stability (Tanford, 1962; Brandts, 1964; Kawaguchi & Noda, 1977) in a manner described previously (Nall & Landers, 1981). Comparison of columns two and three in Table I suggests that it may be possible to predict overall trends in stability for a series of homologous proteins although considerably more data are required in order to test the limits of this hypothesis.

Functional Dependence of Relative Amplitudes and Time Constants on Guanidine Hydrochloride Is Consistent with a Three-State Model. Hagerman (1977) has developed a formalism for testing the consistency of the observed kinetic pattern with a simple three-state mechanism:

$$U_1 \overset{\text{slow}}{\rightleftharpoons} U_2 \overset{\text{fast}}{\rightleftharpoons} N$$

U_1 and U_2 are unfolded species in slow equilibrium which are optically indistinguishable (i.e., they have the same fluorescence) while N is the native protein. The approach (Hagerman & Baldwin, 1976; Nall & Landers, 1981) is to use the limiting value of the relative amplitude for refolding and the functional dependence of the time constants on Gdn·HCl concentration to predict the equilibrium transition curve and the Gdn·HCl dependence of the relative amplitude (α_2). The assumed behavior of the time constants is shown in Figure 8 (bottom panel). For a limiting value of $\alpha_2 = 0.7$,

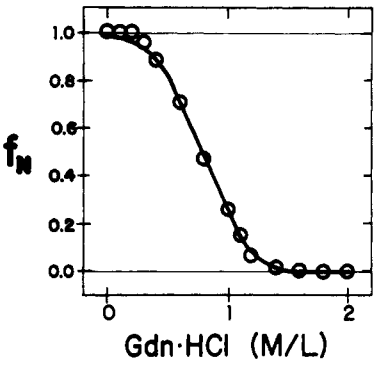

FIGURE 9: Comparison of the observed equilibrium transition curve for iso-1-AM with that predicted from the kinetic data (Figure 8) using a simple three-state model. The observed transition (Figure 6) has been converted to a plot of the apparent fraction of native protein, f_n, by subtracting out the base-line dependence of the fluorescence of the completely unfolded protein. The fluorescence of the native protein has been assumed to be independent of Gdn·HCl concentration.

the equilibrium transition (Figure 9) and relative amplitude behavior (Figure 8, top panel) are predicted. The agreement between predicted and observed behavior is satisfying but does not rule out other, more complex mechanisms.

Relative Amplitude Differs from That Observed for Iso-2. In Figure 3 of the preceding paper (Nall, 1983) the relative amplitude and time constant dependence on Gdn·HCl is presented for iso-2 folding–unfolding kinetics monitored by fluorescence. Comparison with Figure 8 shows that although the time constants exhibit similar behavior, the relative amplitudes (α_2) are different for refolding experiments ending below the transition zone. For complete refolding α_2 for iso-1-AM has a lower value than iso-2 (about 0.9 for iso-2 but 0.7 for iso-1-AM) but, above the transition, rises to one for both proteins.

The difference in the relative amplitude for complete refolding must be attributed to the limited differences in amino acid sequence (Table II). A popular model which allows prediction of the fraction of fast refolding material is the proposal that cis–trans isomerization about proline imide bonds in the unfolded protein produces slow refolding material which must isomerize its proline residues back to the native configuration before folding can occur (Brandts et al., 1975). This model predicts that the fraction of fast phase increases as the number of prolines decreases. The opposite is observed in the present case. Iso-1 and iso-2 contain four proline residues at homologous positions, but iso-2 contains an additional proline in the N-terminal region not found in iso-1 (Table II).

It should also be noted that the difference in α_2 for refolding for the two proteins holds only for folding monitored by fluorescence changes. For iso-2 the relative amplitude for complete refolding is different when folding is monitored by different structural probes [Figure 3, top panel (Nall, 1983); Nall & Landers, 1981]. The relative amplitude behavior of iso-2 when monitored by absorbance is essentially the same as that of iso-1-AM monitored by fluorescence.

On the other hand the sequence differences must be compatible with the similar kinetic behavior of the two proteins. This may indicate that the kinetic pattern of folding reflects the degeneracy in the way sequence information codes for tertiary structure in homologous proteins.

Conclusions

We have specifically blocked a free sulfhydryl group on iso-1 and have found that the modified protein, iso-1-AM, is indistinguishable from iso-1 by several physical and functional criteria. The Gdn·HCl-induced unfolding of iso-1-AM has been shown to be reversible, and the kinetic properties are in most respects similar to those of homologous cytochrome *c*'s. The one exception is that α_2 below the transition zone is smaller for iso-1-AM than for iso-2. This minor difference is in some way related to the rather conservative differences in primary structure.

Table II: Location of Primary Structure Differences between Iso-1-AM and Iso-2 Cytochrome *c*[a]

change: iso-1 → iso-2		
N terminus	surface	buried
– → Ala(–9)	Leu(15) → Gln(15)	Val(20) → Ile(20)*
– → Lys(–8)	Lys(22) → Glu(22)	Leu(98) → Met(98)
– → Glu(–7)	His(26) → Asn(26)	Cys-AM(102) → Ala(102)*
– → Ser(–6)	Ala(43) → Val(43)	
Glu(–4) → Gly(–4)	Glu(44) → Lys(44)	
Ala(–1) → Pro(–1)	Lys(54) → Asn(54)	
	Leu(58) → Lys(58)	
	Asn(62) → Asp(62)	
	Asn(63) → Ser(63)	
	Gly(83) → Ala(83)	
	Lys(99) → Thr(99)	
	Glu(103) → Lys(103)	

[a] Residues are classified according to the location of homologous residues in tuna cytochrome *c* (Dickerson & Timkovich, 1975). The numbering system is that of horse cytochrome *c*. The additional N-terminal residues found in the yeast proteins have been assigned negative numbers and placed in a category of their own since their location in the tertiary structure (buried or surface) is not known. Residues that are only partly buried are indicated by an asterisk. Primary structures are from Narita & Titani (1969), Stewart et al. (1966), and Lederer et al. (1972) and from DNA sequencing of the genes for iso-1 (Smith et al., 1979) and iso-2 cytochrome *c* (Montgomery et al., 1980).

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Proteolytic Activity of α_2-Macroglobulin–Enzyme Complexes toward Human Factor VIII/von Willebrand Factor†

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ABSTRACT: The low level of enzymatic activity of certain α_2-macroglobulin–proteinase complexes could be important to the function of factor VIII/von Willebrand glycoprotein since it is especially sensitive to proteolytic cleavage. To test this possibility, complexes of α_2-macroglobulin with plasmin, trypsin, and thrombin were formed in at least a 2:1 molar ratio of α_2-macroglobulin:proteinase and tested for effects on the factor VIII procoagulant activity of the factor VIII/von Willebrand glycoprotein. Neither the α_2-macroglobulin–trypsin complex nor the α_2-macroglobulin–plasmin complex affected factor VIII procoagulant activity. The behavior of the α_2-macroglobulin–thrombin complex was different. When α_2-macroglobulin and thrombin were incubated in a mole ratio of 3:1 or less, factor VIII procoagulant activity was enhanced to about the same extent as with free thrombin. Even at a 24:1 mole ratio, the mixture could produce 45% of the increase in factor VIII activity obtained with free thrombin. The isolated α_2-macroglobulin–thrombin complex could also activate the factor VIII procoagulant function to about 45% of the level obtained with an identical amount of uncomplexed thrombin. Analysis of the α_2-macroglobulin–^{125}I-labeled thrombin complexes by rechromatography or by polyacrylamide gel electrophoresis in sodium dodecyl sulfate indicated that this activation was not due to free thrombin. We conclude that the α_2-macroglobulin–thrombin complex retains sufficient proteolytic activity to activate the procoagulant function of factor VIII/von Willebrand glycoprotein despite the latter being a very large substrate, having an estimated molecular weight of 1–20 million.

Proteinases appear in the circulation during blood clotting, fibrinolysis, or activation of the complement system. These proteinases can be inactivated by one or more of the several proteinase inhibitors in blood (Muller-Eberhard, 1975; Laurell & Jeppson, 1975; Heimburger, 1975). The present study focuses on α_2-macroglobulin, which is normally present in plasma in a concentration of 2–4 mg/mL (Laurell & Jeppson, 1975). This inhibitor is a large protein composed of four identical subunits and has a molecular weight of 725 000 (Swenson & Howard, 1979). The observation that α_2-macroglobulin binds proteinases and is cleaved by them led to the formulation of the "bait-trap hypothesis" in which the proteinase cleaves α_2-macroglobulin and then becomes entrapped as conformational changes occur (Barrett & Starkey, 1973). Certain proteinases remain bound even after the dissociation of α_2-macroglobulin into half-molecules in 4 M urea; under these conditions 2 mol of chymotrypsin remain bound per mol of α_2-macroglobulin (Pochon et al., 1978). More recent studies have shown that the strength of binding of α_2-macroglobulin to different proteinases varies widely; for example, analyses of α_2-macroglobulin–trypsin and α_2-macroglobulin–papain complexes show that 61.2% of the trypsin, but only 8.3% of the papain, becomes bound with a stability indicative of a covalent bond (Salvesen & Barrett, 1980).

Previous studies suggest that α_2-macroglobulin–proteinase complexes may retain low levels of proteolytic activity against

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