TRANSIENT CONFORMATIONAL STATES IN PROTEINS FOLLOWED BY DIFFERENTIAL LABELING

Charis Ghélis, *Laboratoire d'Enzymologie Physico-Chimique et Molecularie, Universite de Paris XI, 91405 Orsay, France*

ABSTRACT Refolding of previously denatured and reduced elastase has been followed by titration of chemical reactivities of amino acid side chains to study the topography of the protein in the native state, and the microenvironment variations of protein side chains during the structural transition. Groups accessible to chemical reagents in the denatured form and buried in the "native" form were used as a local conformational probe. Times of labeling, depending on the reagent used, ranged from 100 to 800 ms. The reaction was stopped by isotopic dilution with an excess of unlabeled reagent under denaturing conditions to obtain a chemically homogeneous but heterogeneously labeled material. Peptide fractionation after degradation of the labeled proteins allowed the determination of the amount of radioactive label incorporated by the individual side chains during the refolding. Refolding rates, determined by physicochemical, enzymatic or immunochemical criteria, were compared with the conformational states of protein areas and evaluated by the variation of chemical reactivity at various denaturant concentrations. The importance of the last folding stages is emphasized by the results obtained which indicate that early during the refolding, two domain substructures (H-40 to H-71 and M-180 to H-200) are stabilized, while the protein remains inactive at the time ranges of the labeling reactions.

INTRODUCTION

Rules for the structural organization of proteins have been discussed (Richards, 1977; Schulz, 1977; Chothia, 1975, 1976) and pathways involving sequential processes in protein folding have been proposed and reviewed (Wetlaufer and Ristow, 1973; Karplus and Weaver, 1976; Anfinsen and Scheraga, 1978; Baldwin, 1975; Creighton, 1978). Nucleation of limited regions of the polypeptide chain is generally proposed as a first step of the process (Wetlaufer, 1973; Matheson and Scheraga, 1979). The growth around the "nucleus" is considered the driving force to the functional structure, through substructures and stabilization of domains (Wetlaufer, 1973; Schulz, 1977; Yon, 1978). Experimentally, the detection and characterization of intermediary states or structured segments of the polypeptide chain are approached by various methods: (*a*) Immunochemical techniques (Furie et al., 1975; Teale and Benjamin, 1976; Chavez and Scheraga, 1977; Creighton et al., 1978). (*b*) Kinetic methods (Garel and Baldwin, 1975; Baldwin, 1975). (*c*) Nuclear magnetic resonance techniques (Wüthrich and Wagner, 1978; Wagner and Wüthrich, 1978; Snyder et al., 1975) and protein hydrogen exchange coupled with infrared spectroscopy to detect structured intermediates during the refolding (Englander et al., 1972). (*d*) Accessibility to proteolysis (Burgess et al., 1975). (*e*) Analysis of the rates of formation of disulfide bonds (Anderson and Wetlaufer, 1975, 1976; Acharya and Taniuchi, 1976, 1977) or by (*f*) Quenching of disulfide formation during the folding of protein structured by disulfide bonds for trapping intermediates and determining kinetic pathways (Creighton, 1974, 1975, 1978, 1979).

This paper presents data obtained by differential labeling on the refolding of denatured and reduced elastase. The approach proposed by Hartley (1970) to evaluate ionization constants of amino groups in proteins (Kaplan et al., 1971) was adapted to detect local conformational changes during the alkaline transition of δ-chymotrypsin (Ghélis et al., 1975).

As the accessibility and the microenvironment of a large number of groups are modified during protein refolding, the apparent chemical reactivity of amino acid chains can be used as an intrinsic probe of the conformational state of the polypeptide chain.

The topography of the elastase molecule was studied by measuring side-chain chemical reactivities of the native and denatured-reduced protein in intermediary denaturant concentrations.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]acetic anhydride and [^{3}H]acetic anhydride were from the Radiochemical Centre Ltd. (Amersham, England). [^{14}C]dimethyl sulfate and [^{14}C]iodoacetic acid were obtained from NEN Chemicals GmbH. The 2-nitro-5-thiocyanobenzoic acid and elastin-orcein used was from Serva Feinbiochemical. (GmbH & Co., (Heidelberg, Germany). *N*-acetyl-*L*-trialanyl-methylester was purchased from Sigma Chemical Corp. (St. Louis, Mo.). Trypsin, crystallized thrice and salt-free, was purchased from Worthington Chemical Corp., (Freehold, N.J.). Aminopeptidase M was from Sigma Chemical Corp., and carboxypeptidase C from Rohm GmbH (Germany). All other chemicals were of analytical grade.

Protein Preparations

Pig pancreatic elastase (E C 3.4.4.7) was purified from an acetonic extract of porcine pancreas according to a method adapted from Shotton (1970). The final protein preparation was purified and characterized by filtration on a Sepharose-antielastase affinity column, as described by Ghelis et al. (1978). [^{14}C]acetylated elastase, modified on the three lysine residues, was prepared by reaction with [^{14}C]acetyl-benzotriazole (Reboud-Ravaux and Ghélis, 1976) and used for the radioimmunoassay of antielastase binding to renatured elastase; it was checked by double immunodiffusion. [^{14}C]acetylated elastase had antigenic activity identical to the native protein.

Anti-elastases specific to the native form of the protein were prepared by hyperimmunization of rabbits. Fractionation of antibodies specific to delimited areas of elastase molecule was performed on affinity columns of Sepharose to which elastase or elastase fragments were covalently linked.

Binding of Renatured Elastase to Anti-Elastases: Radioimmunoassays

Antigenic activity of renatured elastase was determined by competitive displacement of [^{14}C]-acetylated-elastase from the antielastase-[^{14}C]-acetylated-elastase complex. The assays were performed with [^{14}C]acetylated protein (specific radioactivity 15 μC_i/mmol) in concentrations of 5–30 μM in phosphate-buffered saline, pH 7.0, 25°C. The incubation time ranged from 20 min to 3 h.

Protein concentration of elastase solutions was determined spectrophotometrically at 282 nm with a Cary 118 spectrophotometer (Cary Instruments, Fairfield, N.J.), using a molar extinction coefficient of $\epsilon = 5.4 \times 10^4\ M^{-1}\ cm^{-1}$. The protein concentration of the elastase derivatives and fragments was based on their amino acid composition determined after hydrolysis.

Enzymatic Activity Measurements

The enzymatic activity of elastase was determined either titrimetrically by following the hydrolysis of a synthetic substrate (Ac-[Ala]$_3$OMe) in a EIL-Vibron pH meter or by the standard method proposed by Naughton and Sanger (1961) to follow the digestion of elastin.

Radial immunodiffusion was used to quantify the amount of antigen-antibody complex formed during the renaturation experiments.

Denaturation and Reduction of Elastase

Native as labeled elastase was denatured in 6 M guanidinium chloride solution, in 10 mM acetate buffer, pH 5.5, to avoid protolysis.

Protein disulfide bridges were reduced in 100 mM β-mercaptoethanol in 50 mM of a borate buffer, pH 8.0, 25°C, for 4 h. Then the pH was readjusted to 5.5. The reducing agent was eliminated on a Sephadex G 25 column equlibrated in 6 M GuHCl, pH 5.5, and the free sulfhydryl groups titrated by using DTNB reaction (Ellman, 1959).

Renaturation of the Reduced and Denatured Protein

Renaturation was performed by simple dilution to a final concentration of 400 nM elastase, in 500 nM denaturing agent, at pH 5.5, in 20 mM acetate buffer.

In some experiments, a step dialysis was used for the complete elimination of the reducing and denaturing agents, at 4°C, overnight.

The recovery of characteristic optical signals, chemical reactivity of amino acid side-chains, and antigenic and enzymatic activities have been used as a measure of the rate and extent of renaturation.

Structural Characterization of Reduced-Denatured and Native Elastase

UV absorption difference spectra were obtained with a Cary 118 spectrophotometer with a cell holder thermostated at 25°C and at the appropriate pH values.

Circular dichroism spectra were recorded with a Jouan-Jobin Yvon III dichrograph in a cell thermostated at 25°C.

Fluorescence measurements were carried out with a spectrofluorimeter (Perkin-Elmer Corp., Norwalk, Conn.; model MPF-44B).

Differential Labeling Technique

A pulsed quenched flow apparatus was used to titrate chemical reactivities of amino acid side-chains at different denaturant concentrations. The apparatus used was a Durrum multimixing system (Durram Instrument Corp., Sunnyvale, Calif.; model (D-132) modified in order to use sets of different volume syringes. Denatured-reduced protein is incubated in syringe I and thermosttated at 15°C. Syringe II contained the labeling reagent in the appropriate concentration of denaturant to give the intermediary denaturant amount. The two solutions were mixed as the plunger drove them through a mixing chamber, and then isotopic dilution occurred upon mixing with the solution of unlabeled reagent from the quenching syringe into a second mixing chamber. It was possible, by varying the length of tubing between the two mixing chambers and the driving pressure, to perform labelings from 100 to 800 ms. The labeling reagents were | ^{14}C |-acetic anhydride for amino groups modification (followed by hydroxylamine treatment); | ^{14}C |-dimethyl-sulfate for the modification of tyrosyl groups; | ^{14}C |-iodoacetate for methionine residues; and | ^{14}C |-ethoxyformic anhydride for histidine residues. In this last case a back-titration of the modified histidine residues was performed, as described by Tenu et al. (1976).

The amount of label incorporated into the side-chains in the denatured form was considered as the normal chemical reactivity of an individual group. The apparent chemical reactivity of an individual amino acid side chain was expressed, in our experiments, as:

$$r = \frac{\text{specific radioactivity in the "structured" protein}}{\text{specific radioactivity in the denatured protein}}.$$

Specific radioactivities were determined with a Packard liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) and were corrected for quenching by the protein solutions.

Preparation of the Peptides from the Labeled Protein

Labeled protein degradation was performed either by proteolysis, using an immobilized-trypsin preparation (Affigel-10-trypsin, prepared in the laboratory) or by chemical degradation after reduction

of the disulfide bonds, using 2-nitro-5-thiocyanobenzoic acid (Degani and Patchornik, 1974). The β-thiocyanoalanine proteins were cleaved in high yield by treatment at alkaline pH (Jacobson et al., 1973).

Analytical Peptide Mapping

Peptide separations were performed on a Bondapak C_{18}/Corasil column (2 × 610 mm, Waters Associates Inc., Milford, Mass.) at room temperature and column pressures ranging from 500 to 1,000 psi. A linear gradient was used from 100 mM acetate (solvent A) to acetonitrile (solvent B), at flow rate of 1 ml/min. The elution of peptides was monitored in continuous flow by reaction with fluorescamine, and by measurement of the radioactivity after mixing with an aliquot of Bray solution.

End Group and Amino Acid Analysis

Dansyl amino acids were separated by thin-layer chromatography (Spivak et al., 1971) and their concentration determined by spectrofluorimetry, according to Gros and Labouesse (1969).

Amino acid analysis after 24 h hydrolysis in 3 N *p*-toluene-sulfonic acid, was performed in a Kontron amino acid analyzer (Durrum DC-4 resin), equipped for the fluorimetric detection of amino acids. The reagent used was *o*-phthalaldehyde.

RESULTS

Renaturation Kinetics of Denatured and Reduced Elastase

Renaturation rates of denatured and reduced elastase are presented in Fig. 1. The denatured and reduced enzyme (20 μM, 5M GuHCl, 100 mM β-mercaptoethanol, pH 8) was renatured by dilution to 400 mM GuHCl, 10 mM acetate buffer, pH 5.5, and 1 mM GSH-GSSG. The

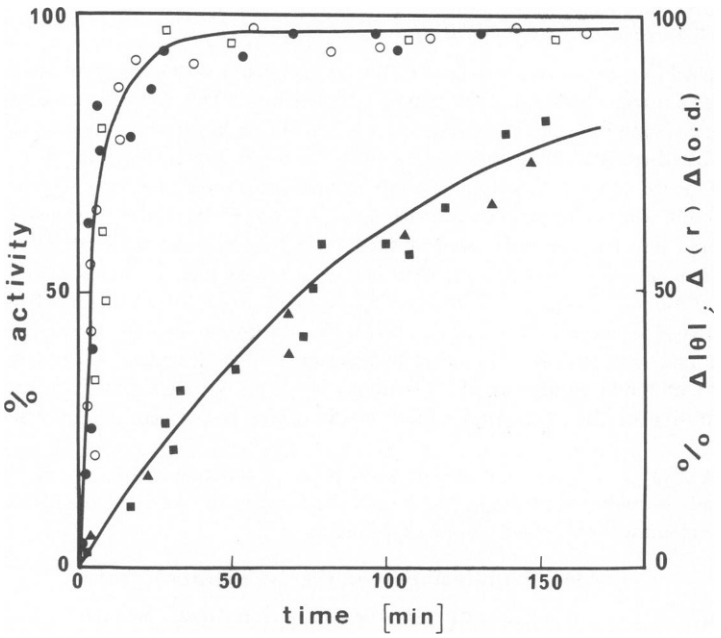

Figure 1 Rates of renaturation of reduced and denatured elastase. (5 M GuHCl, 1mM GSH-GSSG.) The process was followed by the reappearance of antigenic (■) and enzymatic (▲) activities and also by the variation of optical signals:molar ellipticity (235 nm, ●); differential absorption (282 nm, □). Chemical reactivity (O) determinations represent values obtained for the rates of "masking" side-chains of residues valine-16, methionine-180 and tyrosine-234. As the determined rates are similar, the same symbols were used for the three residues to simplify the figure. The protein concentration during the renaturation was 0.1–2 μM in 10 mM acetate buffer, pH 5.5, at 15°C.

final concentration of enzyme was on the order of 2 μM. The overall conformational properties of elastase molecule are recovered rapidly and before the return of the enzymatic and antigenic activities. The antigenic properties of the protein were followed in this experiment by using inhibiting antibodies, measuring the binding ratio of native [^{14}C]-acetyl-elastase, and refolding unlabeled enzyme to antielastase (Ghélis et al., 1978).

Titration of the chemical reactivity of three amino acid side-chains V-16, Y-34, and M-180, in conditions described in Materials and Methods, indicated the same rapid kinetics as for the formation of the overall conformation (Fig. 1). To analyze more precisely the structural rearrangements during the refolding, chemical reactivity of elastase individual side-chains was determined in the native and denatured form and the obtained values were used as a local conformational probe.

Titration of the Chemical Reactivity of Amino Acid Side-Chains in Native Elastase

The values obtained for the apparent chemical reactivity of individual side-chains in the native form of pancreatic elastase are illustrated in Fig. 2. Labeling was performed as described in Materials and Methods and the reaction time, depending on the reagent used, ranged from 100 to 800 ms. The data show essentially two regions in the native form of the protein, including residues with decreased chemical reactivity: region I from H-40 to H-71, and region II from M-180 to H-200. The two methionine residues of elastase seem to be buried, as reported previously by Jori et al. (1974). A localized decrease in the apparent

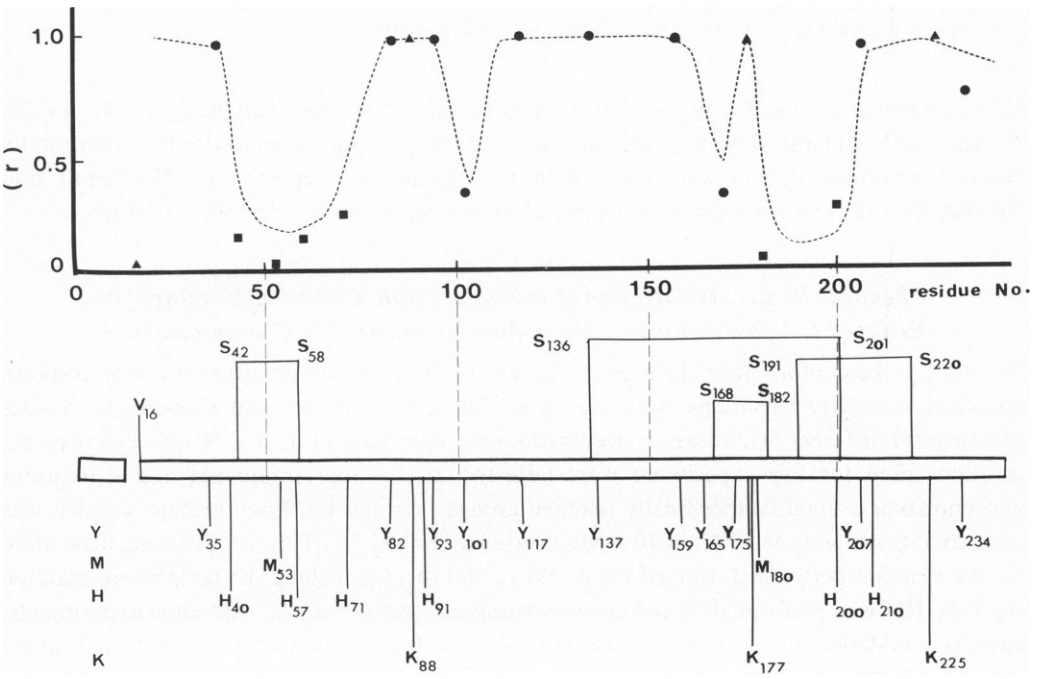

Figure 2 Chemical reactivity (r) of individual amino acid side-chains of elastase in the native form of the enzyme. The reagents used were [^{14}C]-acetic anhydride (▲), [^{14}C]-dimethyl sulfate (●), and [^{14}C]-iodoacetate (■). The reference value for a totally accessible group was the reactivity determined for each residue in the denatured and reduced form of the protein. Each assay was made in triplicate. The lower part of Fig. 2 gives all the side-chains modified during the differential labeling by the corresponding reagent.

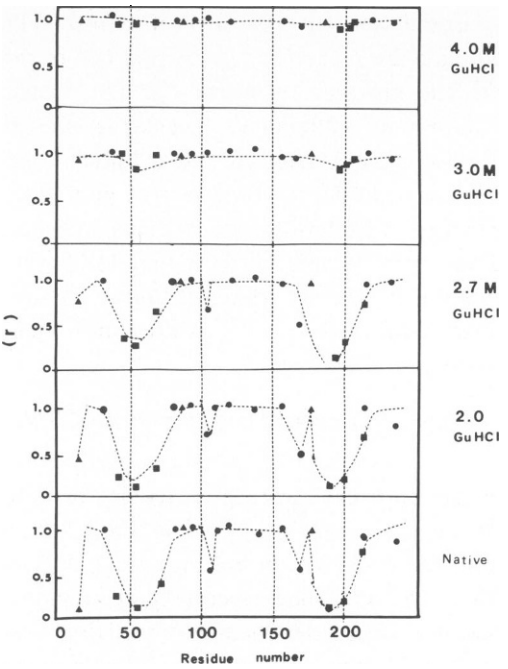

Figure 3 Titration of the chemical reactivity (r) of elastase side-chains during its refolding, in varying denaturant concentrations. (0–4 M GuHcl). Labeling time in this experiment was 400 ms. The concentration of denatured and reduced elastase was 4 μM, 10 mM acetate buffer, pH 5.5. The symbols used are those of Fig. 2. More details are given in Materials and Methods.

chemical reactivity was found for V-16 (Kaplan et al., 1971; Karibian et al., 1974). Y-101, Y-165, and Y-234 also showed decreased chemical reactivity in contrast with the other totally reactive tyrosines and in agreement with the observation reported by Gorbunoff and Timasheff (1972) for the existence of three classes of reactivity for elastase tyrosines.

Chemical Reactivity Titration of Individual Side-Chains of Denatured and Reduced Elastase in Various Guanidine Hydrochloride Concentrations

To analyze the conformational states of elastase in intermediate denaturant concentrations, chemical reactivity variations were evaluated for several amino acid side-chains. Values obtained for different denaturant concentrations are reported in Fig. 3. |^{14}C|-labeled reagents solutions used for this experiment were adjusted at the appropriate pH and denaturant concentration to modify specifically reactive groups (syringe II). Each protein solution was incubated (syringe I) at 15°C, in 10 mM acetate buffer, pH 5.5. Times of labeling, depending on the reaction performed, ranged from 100 to 800 ms. Quenching of the labeling reaction (syringe III) was performed by the corresponding unlabeled reagent. For some experiments, such as acetylation and carboxymethylation, |^{3}H|-labeled reagents were used for the isotopic dilution instead of "cold" reagent, to incorporate a tracer for the detection of peptides (Bosshard et al., 1978).

The data presented here indicate that in distinct areas of elastase molecule during its refolding, the chemical reactivity of several side-chains decreases rapidly to values corresponding to buried amino acids. These results suggest that, even at 3 M GuHCl concentrations, structural elements of the chain are stabilized.

DISCUSSION

Several experimental approaches have been proposed to demonstrate the existence of a sequential pathway in protein folding. One of the most direct evidences was provided by Creighton (1974, 1976, 1978) who trapped and analyzed the intermediary species formed during the refolding of bovine pancreatic trypsin inhibitor or ribonuclease. Another approach is analysis of the process by a kinetic study of the return of structural and functional properties of the molecule, using local conformational probes.

During the refolding of denatured and reduced elastase, structural and functional properties reappear with different rates: slow processes are critical for the return of enzymatic activity (Fig. 1). Several events, such as structural rearrangements or disulfide pairing, can explain the slow return of functional properties. The search for the right disulfide pairing seems an independent process, since the overall conformation and the "masking" of side-chains are rapidly reached.

A plausible assumption may be that two structural domains of the protein are folded independently from different nucleation sites; they then have to interact in a right way to generate enzymatic activity (Ghélis et al., 1978). The interaction between domains and the rearrangements, small in amplitude but significant, which arise from these interactions, might be slower. Some arguments are presented which support the assumption that full expression of the activity for a multidomain protein is a consequence of the inter-domain interaction (Ghélis and Yon, 1979).

Experimental data presented here, based on the chemical reactivity of amino acid side-chains of the enzyme in various denaturant concentrations, are consistent with the hypothesis that elastase achieves a domain-like structural organization at denaturant concentrations and pH where the protein is inactive and disulfide pairing is excluded. It is important to emphasize that the structural elements characterized by short-time labeling (100–800 ms) are not necessarily the same as conformational states stabilized in equilibrium at a given concentration of denaturant.

The method of differential labeling presented here can be used to study the topography of native proteins. Internal groups in the native form of the molecule are not labeled by the reagent in the absence of denaturant, even for times of labeling of several seconds. This allows a characterization of the accessible and buried surfaces of the protein by using different reagents.

Measurement of the chemical reactivity of amino acid side-chains is a potent tool in the study of protein folding, following the process in different parts of the polypeptide chain in protein concentrations ranging from 1–5 nM.

The data obtained for elastase are not sufficiently detailed to allow a comparison with the crystallographic results available for this protein. Nevertheless, the accessibility of side-chains to chemical reagents follows a regular curve (dashed lines of Figs. 2 and 3) in which two buried areas are well delineated: they correspond to the structural domains of the protein, as observed in the three-dimensional structure (Sawyer et al., 1978).

A kinetic study of elastase refolding after denaturation and reduction is now under investigation. The nature of early stabilized substructures, determined by differential labeling in the experimental conditions described in this paper, is analyzed in comparison with predicted nucleation sites (Matheson and Scheraga, 1978) for elastase and the other homologous serine proteases.

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DISCUSSION

Session Chairman: F. M. Richards *Scribe*: Michael Brenowitz

MARTIN: Elastase is a calcium protein. Have you controlled the calcium concentration?

GHÉLIS: No, not at all. The calcium concentration has an effect on the expression of the function that is on the activity of elastase. I don't think this is important for the stabilization of the structure.

MARTIN: Terbium replaces the calcium in elastase and gives an enhancement factor of ~10,000—the highest enhancement factor of any situation we have encountered. The enchancement is presumably due to energy transfer from tryptophan 141 to terbium. It would be an interesting experiment to follow the rate of return of terbium luminescence on renaturation and compare it with return of enzyme activity.

GHÉLIS: Starting from the native form, yes. There are conformational sites for calcium or terbium in the native state. I don't know if there are calcium sites in the denatured form.

SIMON: A number of years ago, Bill Koningsberg, Keith Moffat and I examined the reactivity of oxy- and deoxy-hemoglobin with a number of maleimides and we observed that with some of these maleimides not only did the reaction rate change as a function of ligation but, more significantly, the stereochemical specificity of the reaction of the maleimides across the C-C bond was influenced by the detailed chemistry of the particular maleimide we employed. One must exercise caution in using chemical modifying reagents as probes for structure; one should recognize the possibility that the specific chemical properties of the reagent employed, as well as specific local conformations of the protein in the vicinity of the reactive amino acid, may influence the reaction rate.

GHÉLIS: Yes, I agree. In this particular case the reagents used are of low specificity. They are acylating reagents,

acetic anhydrides, or iodoacetate. The time of reaction is ~20 ms so that there is no lag time to induce a conformational readjustment by the reagent or a limitation by the stereochemistry of the reagent.

SIMON: An ideal test for this kind of thing is the use of multiple reagents which have the same effect to test to see if they are all equally influenced by the protein conformation.

WÜTHRICH: It has often been observed that unfolded forms of proteins are considerably less soluble in aqueous media than the folded forms, and also that they tend to aggregate. To what extent could intermolecular interactions affect your observations? What controls do you have to be sure the accessibility of the residues in the open chain forms of the protein is not affected by intermolecular aggregation?

GHÉLIS: A variation of chemical reactivity in the denatured form was tested in the presence of model compounds, which were derivatized amino acids. The order of the reactivities was the same. I agree with Dr. Wüthrich's comment. The aggregation depends on the concentration of the protein. In this case the concentration ranged from 1–2 nm, a very low protein concentration.

HANTGAN: I would like to comment on the general question of the sequential vs. random pathway of protein folding and in RNAse. The differential labeling approach has been successfully applied to the refolding of thermally denatured RNAse and has been interpreted as favoring a nucleation-directed pathway. But there is an equally large body of evidence that if you start with the reduced protein the pathway of folding is not so directed.

GHÉLIS: I have insufficient data on the time process and the evolution of the chemical reactivity as a function of the time of refolding to respond to your comment.

WETLAUFER: I would like to be a little bit clearer about the description of the experiments, particularly those comparing different concentrations of GuHCl denaturant. Can you describe them a bit more? What is the T_o, initial stage, and then what solutions do you mix?

GHÉLIS: In the multi-mixing system (see Fig. 3) we have 4M GuHCl and the protein diluted as a first step in a solution containing 4M GuHCl and the labeling reagent. 10 ms later the isotopic dilution takes place. For 2M GuHCl we have the protein in 4M GuHCl diluted at the first step in 2M GuHCl plus the labeling reagent; 20 ms later the isotopic dilution takes place. This occurs at pH 5 and in some cases in the presence of reducing agents.

NÉMETHY: As I understand your conclusions, the reduced activity in two places in the sequence corresponds to the formation of two domains prior to the final formation of the structure. How do you know from these experiments that you have two separate domain foldings rather than an overall incorrect folding which already contains reduced accessibility of some groups which come to the interior of the protein?

GHÉLIS: By proteolytic digestion of the enzyme we can obtain two fragments corresponding to the structural domains. After chromatographic separation, we observe that the isolated domain can refold and after complementation has the antigenic activity of the enzyme and enzymatic activity compared with non-digested elastase. This indicates that we can isolate two independently folding structural domains. These can reassociate and can give all the structural signals of the native protein, such as circular dichroism and differential absorption spectra. If we observe not the structural signals, but the enzymatic and antigenic activity at denaturant concentration, we can see that in particular denaturant concentrations we have a minimum of refolding for the reassociated domains. It was suggested that with two completely folded domains the problem of the lack of reactivation is due to a wrong conformational coupling between the two domains.

NÉMETHY: Have you thought of doing differential activity experiments on some separate domains? On refolding you ought to get some reduction in reactivity even if you're looking at the domains. Is that feasible?

GHÉLIS: There is about 5%, a very weak activity.

NÉMETHY: Even if you lose some activity it would be some indication that there is some reduced accessibility.

F. RICHARDS: Just one comment from the chair before we continue. You are proposing that there is a collapse of the structure to some undefined globule which then reorganizes without expansion. This is equivalent to taking the pieces of a Chinese wooden puzzle and throwing them into a bag and then asking them to reassemble. I think the experiments are beautiful, but I don't know what to do with the conclusions.

KIM: Work from Robert Baldwin's lab at Stanford has suggested the accumulation of intermediates in the refolding

of oxidized ribonuclease. So there is evidence for the sequential folding process mentioned by Dr. Hantgan in oxidized as well as reduced RNAse. Some details of the intermediates are presented in our poster (Kim and Baldwin, this volume). There is evidence for a quasi-native intermediate which appears to be folded but has at least one proline residue in the wrong cis or trans conformation and another early folding intermediate. Dr. Ghélis you mention in your paper that you use labeling times of 200–800 ms, and you also mention that in the native protein you can use labeling times up to several minutes without observing significant changes. Is it possible to use longer labeling times with one of the kinetic intermediates along the kinetic folding pathway? I ask this because then you could leave the label in for a longer period of time and see if there is a major structural rearrangement which would then expose some side chain groups later on in the folding pathway.

GHÉLIS: I think it is necessary to use a short labeling time. The labeling reaction must be of 10 or 20 ms to avoid any additional reaction during the conformational changes. The time limit is also imposed by the very low concentration of the labeling reagent.

KARPLUS: In light of Dr. Richards' remarks, I have two questions. One, is it clear that under these various conditions the species that you see, somewhat folded up, are along the normal path of folding? It could be that the structures seen here with various amounts of denaturant are the species that are more or less stable but not the ones that the protein actually goes through when it folds up. This would remove some of the difficulties that Dr. Richards sees. Two, is it clear that the intermediates don't have part of the structure that is the native one?

GHÉLIS: It is necessary to trap and characterize these substructures. The problem is to see how these substructures compare, for different reagents, in refolding time and accessibility to the reagent. Finally, these reactivities have to be compared to the accessibility of the side chains in the native form of the protein.

F. RICHARDS: That matter needs more years of work.

ENGLANDER: Is that bottom frame of your Fig. 3 the fully native labeling pattern?

GHÉLIS: Yes.

ENGLANDER: Then what some of us tend to interpret as an intermediate in the folding has exactly the same labeling pattern as the fully native pattern?.

GHÉLIS: The labeling pattern is the same, but probably not the structural pattern.

ENGLANDER: The apparent result, then, is that early on when you initially throw in the transient pulse of label you already have the fully native labeling pattern?

GHÉLIS: Not for the 3M GuHcL. At pH 5.4 and 3M GuHCl, all the species of the protein are in the denatured form and at 2M GUChL only 50% of the species are in the denatured form. So it is difficult to distinguish the labeling rate between the two forms of the protein.

ENGLANDER: I'm having a little trouble coming to terms with the GuHCl data. There are similar data on nucleation that comes from Baldwin's lab. But leaving aside the GuHCl data, it seems to me that the results for the normal solution refolding show that early in the refolding process you have recovered essentially the native form. This doesn't necessarily speak to the issue of folding intermediates, or nucleation intermediates. The only odd quality you see is that the activity returns much later. Have you considered the possibility that regain of activity could require some rather ephemeral final reaction, not very important as far as the basic globular structure is concerned? For example, as Peter Kim mentioned, perhaps it is simply a cis-trans isomerization which could bring a piece of chain, possibly toward the N or C terminal, up where it eventually joins the active site.

GHÉLIS: It could be.

BUTLER: Part of the confusion here may arise from the combination of the temperal refolding and what may be different equilibrium refolded forms. I think the data are beautiful, but it does seem to me that we are applying words like "first" and "then" as if we were looking at the refolding pathway under totally native and non-GuHCl conditions. We have to be very careful about what one can distinguish. Certain structures are formed which may be close to though not identical to the native. There may well be a sort of breathing which is taking place sufficiently slowly under this particular condition so that not very much label gets in; what appears to have native conformation from the ratio of labeling may in fact not be native. We could be erring when we put a time, as opposed to a reaction end state—for instance, so much GuHCl.

GHÉLIS: The problems are technical. First, in multi-mixing systems it is not possible to obtain a large dilution of GuHCl with the available systems. The other problem is the intrinsic chemical activity and the amount of label incorporated in the protein. It is important to have a short "pulse" to avoid any conformational change during the labeling, but it is also necessary to incorporate enough to count something. And we have two steps: The dilution for the denaturation and then the isotopic dilution to stop the reaction of labeling.

NÉMETHY: I'd like to comment on the question that Martin Karplus raised. It seems to me that if you have a high likelihood of partial refolding into long intermediate structures during the folding process, then it is likely that you would get reduced reactivity of some groups other than those seen in the native structure. Your figures indicate that the same groups lose reactivity during the various intermediates and in the final form. That's not a final proof because obviously it limits this question to whether there are any other local wrong foldings. You're also limited by the resolution of your experiments since you didn't label all groups; some local folding nuclei might form which are not detectable by the reagent. From what we see here there seems to be no evidence of wrong refolding.

LIEBERMAN: An interesting observation results from the fact that you have based your study on the reactivity towards labeling or chemical modification. This bears on some structural questions that have come up related to elastase and serine proteases in general. Have they evolved from a gene duplication? Do they show an as yet not understood structural palindrome? It has has been suggested that the internal folding symmetry that appears in these molecules results from the two β-barrels which would be the result of gene duplication. Such evolutionary barrels would maintain some degree of parallel internal symmetry. This is an argument that has been put forth by Andrew McLachlan but your reactivity and modification profiles support the presence of a structural and now apparently a functional palindrome, antiparallel in structure, in the molecule.

WETLAUFER: I'd like to comment on the question that was raised by Karplus about the conditions you have chosen which I understand are necessary for the way you carry out the experiments, namely to dilute from a high GuHCl concentration to an intermediate concentration of denaturant. Might these conditions not strongly bias the choice of pathways if there are either/or choices that can be made in the development of structure? It may turn out that this is the case.

There is evidence of this in the oxidative folding of lysozyme where we can compare systems which have differences not in solvent, but only in the nature of the oxidant by which the disulfides are formed from thiols. It turns out that there is a difference in population of intermediates that we isolate. The difference is so great that we see two new intermediates in an air oxidation that are not seen at all by our detection methods in the glutathione oxidation.