FEBS Letters 356 (1994) 66–71

FEBS 14853

Surface labeling of key residues during assembly of the transmembrane pore formed by staphylococcal α-hemolysin

Musti Krishnasastry, Barbara Walker, Orit Braha, Hagan Bayley*

Worcester Foundation for Experimental Biology, 222 Maple Avenue, Shrewsbury, MA 01545, USA

Received 4 October 1994; revised version received 24 October 1994

Abstract Structural changes in staphylococcal α-hemolysin (αHL) that occur during oligomerization and pore formation on membranes have been examined by using a simple gel-shift assay to determine the rate of modification of key single-cysteine mutants with the hydrophilic sulfhydryl reagent, 4-acetamido-4′-((iodoacetyl)amino)stilbene-2,2′-disulfonate (IASD). The central glycine-rich loop of αHL lines the lumen of the transmembrane channel. A residue in the loop remains accessible to IASD after assembly, in keeping with the ability of the pore to pass molecules of ~1000 Da. By contrast, residues near the N-terminus, which are critical for pore function, become deeply buried during oligomerization, while a residue at the extreme C-terminus increases in reactivity after assembly, consistent with a location in the part of the pore that projects from the surface of the lipid bilayer.

Key words: Surface labeling; Sulfhydryl modification; Gel-shift electrophoresis; Pore-forming protein; Membrane protein; Toxin

1. Introduction

To examine structural changes that occur when a water-soluble protein assembles into membranes, we have been studying staphylococcal α-hemolysin (αHL). The αHL polypeptide of 293 amino acids is secreted by *Staphylococcus aureus* as a monomer, which forms cylindrical oligomeric pores 1–2 nm in internal diameter in lipid bilayers [1]. A model for the assembly of αHL (Fig. 1A) serves to summarize an expanding collection of experimental data, which includes the results of hydrodynamic studies and chemical crosslinking (the pore is an oligomer [2,3]), circular dichroism spectroscopy (both the water-soluble monomer and the assembled pore are largely β-sheet [3,4]), conformational analysis by limited proteolysis (αHL contains a central glycine-rich loop that becomes occluded upon assembly [3,5]), and mutagenesis (certain mutants accumulate as assembly intermediates [5,6]). Recently, it has been shown that the oligomeric forms **3** and **4** (bold print refers to the structures in Fig. 1A) are both heptamers ([7], and unpublished results). At least two regions of the αHL chain participate in pore formation. First, the central loop penetrates the bilayer upon oligomerization [8] and a segment of it lines the lumen of the transmembrane channel in the fully assembled pore (Fig. 1A, **4**) [9]. Second, the integrity of the N-terminus is necessary for pore function. For example, truncation by even 2 amino acids permits oligomerization, but completely prevents the formation of active pores [5]. Here, we describe additional details of the assembly pathway, which have been obtained by probing the accessibility of key residues to chemical modification with a hydrophilic reagent, 4-acetamido-4′-((iodoacetyl)amino)stilbene-2,2′-disulfonate (IASD).

2. Materials and methods

2.1. In vitro transcription and translation of αHL mutants

Mutated αHL coding sequences were obtained by standard procedures and inserted into a pT7 expression vector [10]. Coupled in vitro transcription and translation (IVTT) was performed with [^{35}S]methionine in a T7 polymerase-dependent system [10]. For hemolysis assays, the final methionine concentration in the IVTT mix was 0.5 mM (0.8 Ci/mmol) to ensure the synthesis of mutant polypeptides at concentrations of 10–50 μg/ml.

2.2. Exhaustive modification by IASD

^{35}S-Labeled cysteine mutants (2 μl IVTT mix) were treated with 1 mM DTT in 0.3 M sodium phosphate, pH 8.5, for 5 min at room temperature and then reacted with 20 mM IASD (Molecular Probes) in a final volume of 10 μl. After 2 h at room temperature, 120 mM DTT (2 μl) was added. After a further 5 min, a portion (2 μl) was removed for analysis by SDS-PAGE, to check that complete modification had occurred (see below). The remainder was examined for the ability to form SDS-stable oligomers or lyse rabbit erythrocytes (rRBC) [5,10].

2.3. IASD modification of αHL cysteine mutants in solution

Each ^{35}S-labeled αHL cysteine mutant (2 μl IVTT mix) was incubated in 0.3 M sodium phosphate, pH 7.4 or 8.5, containing 1 mM DTT, for 5 min at room temperature. IASD was then added to 10 or 20 mM (1 or 2 μl of a 100 mM stock) to give a final volume of 10 μl. After 5 s, 1 min and 60 min, portions of the reaction were removed and stopped by adding DTT to 20 mM, followed by one volume of 2 × electrophoresis sample buffer [11]. This treatment prevents further modification. The samples were then analyzed by electrophoresis in 40-cm long 12% SDS-polyacrylamide gels [11] at constant voltage (160 V) at 4°C.

2.4. IASD modification of αHL cysteine mutants on rRBC membranes

Equal volumes (13 μl) of ^{35}S-labeled αHL cysteine mutant (IVTT mix) and 10% rRBC in 20 mM potassium phosphate, 150 mM NaCl, pH 7.4, containing 1 mg/ml bovine serum albumin were mixed and kept at 20°C for 1 h. The membranes were then recovered by centrifugation at 16,000 × *g* and washed with 5 mM sodium phosphate, pH 7.4. In the case of the non-lytic mutant H35C, the intact cells were lysed by the hypotonic buffer. The membranes were resuspended in 20 μl of 0.3 M sodium phosphate, pH 7.4 or 8.5, and fresh 11 mM DTT (2 μl) was added. After 5 min at room temperature, IASD was added to 10 or 20 mM (4 μl of 65 mM or 130 mM stock). At 5 s, 1 min and 60 min, portions of the reaction mix (6.5 μl) were removed and the excess IASD reacted with 150 mM DTT (1 μl), followed by 2 × electrophoresis sample buffer (7.5 μl). After SDS-PAGE and autoradiography, bands containing oligomers (apparent mass ~200 kDa), which were stable in SDS solution at below 60°C, were cut out of the dried gel and heated in SDS to dissociate the subunits before re-electrophoresis.

*Corresponding author. Fax: (1) (508) 842-9632.
Internet: bayley@sci.wfeb.edu

SSDI 0014-5793(94)01240-7

3. Results and discussion

3.1. Characterization of αHL cysteine mutants

Single-cysteine mutants of αHL were prepared at positions 3, 35, 48, 129, 144, 259 and 292. One rationale was to compare the accessibility of the midpoint of the central loop (mutant T129C) with the N- and C-termini of αHL. Thr-129 lies close to proteolytic sites that become occluded upon assembly [5] and, recently, part of the central loop was shown to line the lumen of the transmembrane channel [9]. For H35C, H48C, H144C and H259C, the rationale was twofold. First, all four histidines had been declared essential for pore-forming activity, based on random chemical modification experiments [12,13]. Second, it had been shown that the integrity of the N-terminus (represented here by positions 3, 35 and 48) is crucial for pore function [5].

The extent of oligomer formation on rRBC by the mutant polypeptides was determined by SDS-PAGE [10]. Pore-forming activity was measured by a hemolysis assay [10]. Limited proteolysis was used to detect possible malfolding of αHL monomers in solution and to determine the accessibility of two sites of proteolysis after assembly on rRBC [5]. The latter are diagnostic for occlusion of the central loop, which occurs early in assembly, and for a final step in pore formation involving the N-terminus ([5], and unpublished results). Mutants with cysteines at positions 3, 48, 129, 144, 259 and 292 were similar to the wild-type protein (WT-αHL) in all respects (Table 1). By contrast, H35C had extremely weak hemolytic activity, although it did form oligomers, albeit to a lesser extent than WT-αHL. As a monomer in solution, H35C, like WT-αHL and the other six cysteine mutants, was cleaved in the central loop upon limited proteolysis with proteinase K. By contrast, when digested on the surface of rRBC, H35C yielded a pattern of fragments that differed from WT-αHL (which is largely undigested) and is diagnostic of monomeric and heptameric assembly intermediates (Fig. 1A, **2** and **3**; [5], and unpublished data). To further explore the sensitivity of the seven positions to modification, the cysteine in each mutant monomer was completely derivatized with IASD in solution, as determined by gel-shift electrophoresis (see below). All the derivatized polypeptides, except H35C–IASD, formed oligomers upon binding to rRBC (Table 1). The mutants with the three most C-terminal replacements, H144C, H259C and T292C, retained high hemolytic activity after modification. Modified S3C and

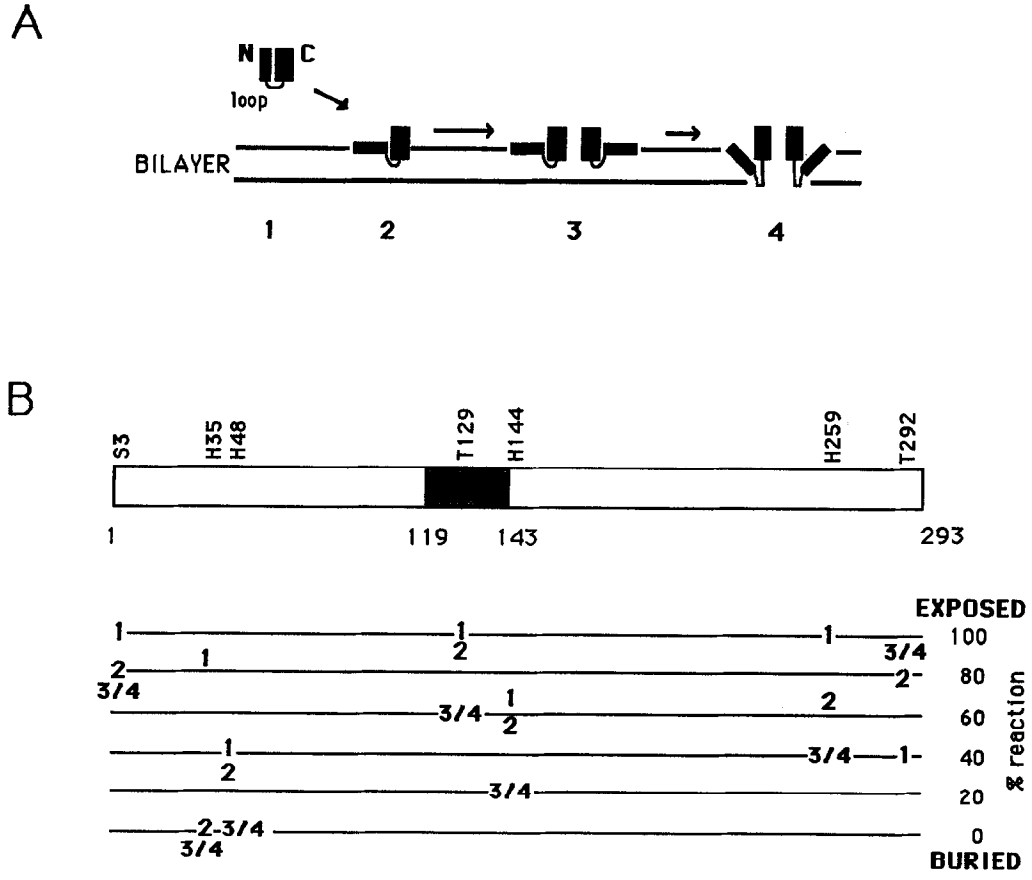

Fig. 1. Summary of surface-labeling experiments on seven single-cysteine mutants of αHL. (A) Working model for the assembly of αHL consistent with earlier findings and the present data. The monomeric polypeptide in solution (structure **1**) comprises two domains linked by a protease-sensitive glycine-rich loop. αHL binds to the cell surface as a monomer, in which the loop is inaccessible to proteinase K (**2**). A non-lytic heptamer is then formed (**3**, shown in cross-section). The subunits further penetrate the membrane to form the lytic pore (**4**), rendering additional sites near the N-terminus inaccessible to proteinase K. Part of the central loop lines the lumen of the pore. (B) Top: positions of single-cysteine mutations in αHL. The central loop is shaded. Bottom: reactivity of the seven cysteine mutants towards 20 mM IASD at pH 7.4. The extent of reaction after 1 min is given, as determined by quantitative autoradiography of dried gels (three determinations, standard deviations within symbol size). Data points are for the monomer in solution (structure **1**), membrane-bound monomer (**2**) and membrane-bound oligomer (**3/4**), and are aligned with the positions of the mutations (top). Forms **3** and **4** are not distinguished in these experiments, but it is known that H35C oligomers become arrested as heptameric prepores, **3**, while the remaining mutants form lytic pores, **4** (see the text).

H48C retained partial activity, while T129C–IASD was almost completely inactivated. Recently, others have reported the properties of αHL with substituted histidines [14,15]. Our findings are in agreement, in that we find that positions 144 and 259 are neither crucial for oligomerization nor for pore activity, while position 48 is discernibly more sensitive. In all three studies, His-35 mutants were found to be highly defective.

3.2. Gel-shift assay for accessibility of cysteine residues

The classical technique of surface labeling can be combined with cysteine mutagenesis, by measuring the reactivity of single-cysteine mutants towards sulfhydryl reagents [16]. In the present study, the standard approach of labeling a 'cold' protein with a radioactive or fluorescent reagent is reversed. Instead, the covalent attachment of IASD to radiolabeled αHL mutants is detected by a rapid gel-shift assay [17] that utilizes standard SDS-PAGE [11]. The magnitude of the shift varies considerably with the position of the derivatized cysteine in the polypeptide chain of αHL. In general, the shift is to lower mobility for monomeric αHL, but to greater mobility for oligomeric αHL.

Comprehensive modification data for all seven mutants are displayed for reactions with 20 mM IASD for 1 min at pH 7.4 (Fig. 1B). Three distinguishable stages in assembly were examined: (i) the monomer in solution (structure **1**, Fig. 1A); (ii) membrane-bound monomer (**2**); and (iii) membrane-bound oligomer (**3** and **4**). Modification with IASD was done after the addition of DTT to ensure that protein sulfhydryls were completely reduced. The reaction was carried out with excess IASD to maintain pseudo first order kinetics, for which the half-life of a reactive sulfhydryl is independent of its initial concentration. The same trends in reactivity were seen over a wide range of reaction times and reagent concentrations. Selected mutants (H35C, T129C and T292C) were also reacted with 20 mM IASD at pH 8.5. The rates of reaction were faster, as expected, but again showed the trends seen at pH 7.4.

Bimolecular rate constants for the reaction of exposed cysteines in proteins with iodoacetamide are in the range 2–5 $M^{-1} \cdot s^{-1}$ (at pH 8.7) [18], which is equivalent to a half-life of 5–15 s in the presence of 20 mM reagent. The half-lives of individual sulfhydryls in the monomeric αHL cysteine mutants in solution at pH 7.4 were in the expected range for exposed residues: ~5 s (S3C, T129C, H259C) to ~1 min (H48C, H144C, T292C), with H35C at an intermediate value of ~30 s. The cysteines at positions 35 and 48 became highly inaccessible during αHL assembly (respectively, ≥ 1000 and 100 times less reactive). While cysteines at positions 3, 129 (the loop), 144 and 259 showed decreases in accessibility upon assembly, they remained relatively reactive. By contrast, the reactivity of the cysteine placed at position 292 (the penultimate residue) was enhanced during assembly. Data supporting these findings and their implications are now considered in greater detail.

3.3. IASD modification of the histidine replacement mutants

The cysteines in all four His→Cys mutants decreased in reactivity as αHL assembled. For example, monomeric H35C

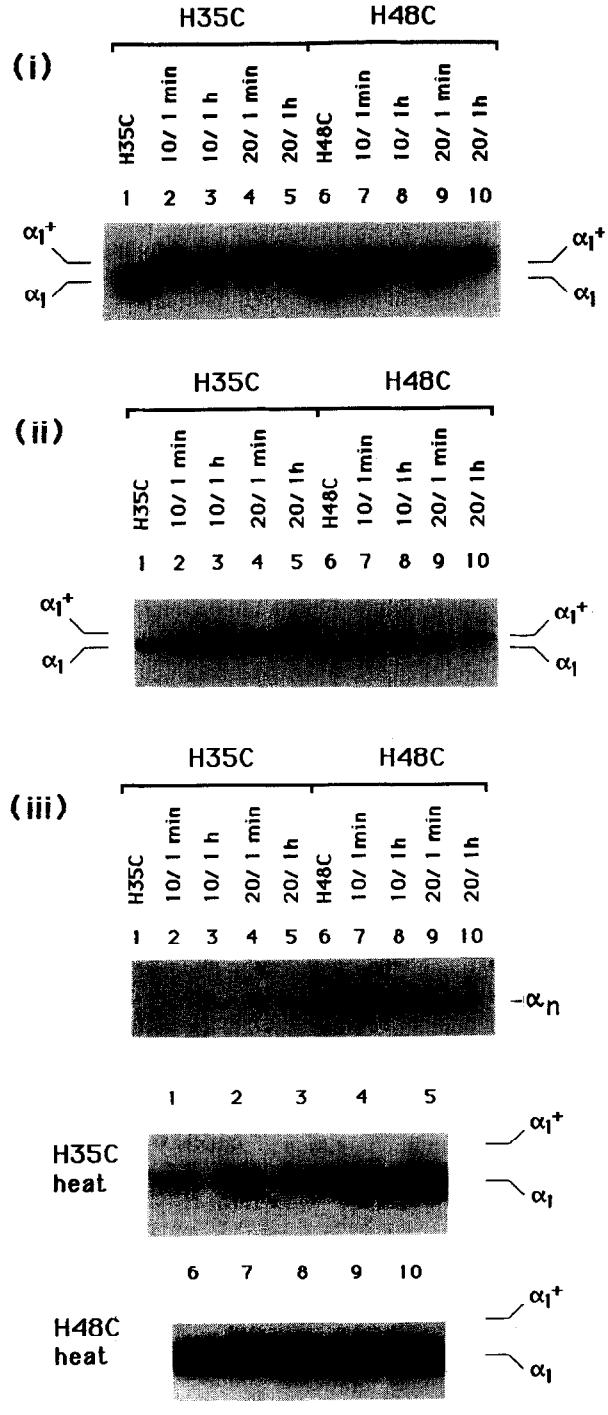

Fig. 2. Comparative reactivity of the sulfhydryls in H35C and H48C at pH 7.4. (i) Monomers in solution. [^{35}S]Methionine-labeled H35C and H48C were treated with 10 mM or 20 mM IASD in solution for 1 min or 1 h. The samples were then subjected to SDS-PAGE. An autoradiogram of a section of the dried gel is shown. α_1, unmodified polypeptide; α_1^+, IASD-conjugated polypeptide. (ii) Monomers on rRBC membranes. ^{35}S-Labeled H35C and H48C were allowed to assemble on rRBC for 1 h at 20°C. Washed membranes were then treated with 10 mM or 20 mM IASD in solution for 1 min or 1 h. After the addition of sample buffer and warming to 45°C, the samples were subjected to SDS-PAGE. Wild-type αHL and many mutant αHL oligomers are stable under these conditions [5,6,10]. An autoradiogram of the bottom part of gel shows monomeric αHL polypeptides. (iii) Oligomers on rRBC membranes. The part of the gel containing H35C and H48C oligomers (α_n) was also subjected to autoradiography (top). The bands were then excised from the dried gel, rehydrated and heated to dissociate the oligomers. Gel slices and supernatants were placed in the wells of a second gel and subjected once more to electrophoresis. Autoradiograms are shown: H35C (center), H48C (bottom).

in solution was completely modified after 1 min with 10 mM IASD at pH 7.4 (Fig. 2(i), lane 2), while membrane-bound monomer showed < 5% modification after 1 h under the same conditions (Fig. 2(ii), lane 3). Membrane-bound H35C oligomers were completely unreacted after 1 h with 10 mM IASD at pH 7.4 (Fig. 2(iii), lanes 3). Oligomeric H35C was also treated with IASD at pH 8.5 and again was completely unreactive. The changes in modification rates were far more dramatic at positions 35 and 48 than they were at positions 144 and 259 (Figs. 1B and 2). This parallels the requirements for these residues for assembly and function ([14,15], and see above). It should be remembered that H35C is defective in pore formation (Table 1) and modification of the oligomer reflects the reactivity of a prepore complex (Fig. 1A, 3). While the lack of reactivity of oligomeric H48C and H35C could be explained by upward shifts in the pK_a values of the cysteines by ≥ 2–3 units [19], it is more likely that Cys-35 and Cys-48 become buried in the lipid bilayer, occluded within individual subunits after a conformational change or hidden at subunit–subunit interfaces within the structure of the oligomer. In the case of H35C, burial in the bilayer or occlusion within the subunit is most likely, as Cys-35 is unreactive in the membrane-bound monomer.

Menestrina and colleagues used diethylpyrocarbonate to modify histidine residues in αHL [12,13] and concluded that all four are required for hemolytic activity. However, assuming that the reactivity of each cysteine parallels that of the histidine it replaces, our findings suggest that diethylpyrocarbonate modifies His-259 of WT-αHL first and that subsequent reaction with the remaining histidines (His-35 > His-144 > His-48, Fig. 1B) results in the observed loss of hemolytic activity (by reaction at His-35 and perhaps His-48, see Table 1) and a slower reduction in the ability to form oligomers (by reaction at His-35). This interpretation is also in keeping with the available data [12,13]. Further, two histidine residues become resistant to modification after assembly into oligomers [12]. Our data indicate that these residues are His-35 and His-48. The open channel of WT-αHL undergoes a transition to a state with 25% higher conductance at low pH [20]. The pK_a for the transition is 5.5, close to the accepted value for the unperturbed imidazole ring of histidine in a polypeptide chain. Because the rate of protonation of the ionizable group is diffusion limited [20], it seems improbable that either the buried His-35 or His-48 is involved. Further, because a bulky substituent can be introduced at positions 144 and 259 with little effect on the activity of αHL, it also seems unlikely that protonation of His-144 or His-259 determines conductance. Therefore, protonation of a carboxylate, the N-terminal amino group or a side chain with a perturbed pK_a value must be responsible.

3.4. IASD modification of the central loop

The central loop of αHL becomes completely resistant to proteolysis when αHL oligomerizes [3,5]. Therefore, when we began this work, we expected Cys-129 in T129C to become resistant to IASD modification upon assembly. In fact, Cys-129, which is highly reactive in the monomer (Fig. 3(i)), remains reactive after binding to membranes (Fig. 3(ii)) and oligomerization (Fig. 3(iii)). This can be explained in the light of a recent finding; part of the central loop lines the conductive pathway [9]. Because molecules of up to 3 kDa pass through the αHL pore [2], it now seems reasonable that Cys-129 in the fully assembled structure can react with IASD (624 Da), while the central loop is not attacked by proteinase K (27 kDa).

3.5. IASD modification at the C-terminus

The penultimate residue 292 in T292C is the only one of the seven cysteines examined here to show an increase in reactivity in the presence of membranes. For example, the sulfhydryl in T292C was far less reactive than that in T129C in monomers in solution and somewhat more reactive after assembly on membranes (Fig. 3(i)–(iii)). To confirm the increased reactivity of T292C upon assembly, IASD modification was carried out on the double mutant S3C/T292C. This was feasible because IASD modification at position 3 produced a larger gel-shift than modification at position 292, and the two monoderivatized forms of the double mutant could be distinguished by SDS-PAGE. Gratifyingly, the observed patterns of derivatization of S3C/T292C, in solution and on membranes, were as expected (data not shown) given the reactivities of positions 3 and 292 in the single-cysteine mutants (Fig. 1B). The C-terminal domain of αHL is involved in the initial aggregation of the subunits on the membrane surface [5]. The high degree of accessibility to solvent in the oligomeric state suggests that the C-terminus may be located in the structure that projects 5 nm or more from the membrane surface in the assembled pore [2,21].

Table 1
Properties of αHL single-cysteine mutants before and after complete modification with IASD

αHL	Untreated				Modified with IASD	
	Oligomer formation	Pore formation	Folding in solution	Conformation on rRBCs	Oligomer formation	Pore formation
WT	++	++	++	4	++	++
S3C	++	++	++	4	++	+
H35C	+	0	++	2/3	0	0
H48C	++	++	++	4	++	+
T129C	++	++	++	4	++	0
H144C	++	++	++	4	++	++
H259C	++	++	++	4	++	++
T292C	++	++	++	4	++	++

Oligomer formation: ++, similar to WT; +, reduced extent of oligomer formation. Protein folding and conformation determined by limited proteolysis: ++, correctly folded as monomer; 2/3 and 4, predominant conformational states on membranes (see Fig. 1A for explanation). Pore formation determined by hemolysis of rRBC in a microtiter assay. The extent of hemolysis after 3 h at 20°C: ++, similar to WT (100% hemolysis in wells containing αHL at 60–240 ng/ml); +, partial lysis in wells containing αHL at ≥ 120 ng/ml; 0, no lysis at up to 4 μg/ml αHL. Other differences were observed. Notably, the lag phases for hemolysis by S3C and H48C were somewhat longer than those of the other unmodified αHL polypeptides. All the IASD-modified αHL polypeptides showed extended lag phases. After 24 h, T129C-IASD gave partial lysis at ≥ 120 ng/ml αHL. Unmodified H35C also gave partial lysis after 24 h, while H35C-IASD did not.

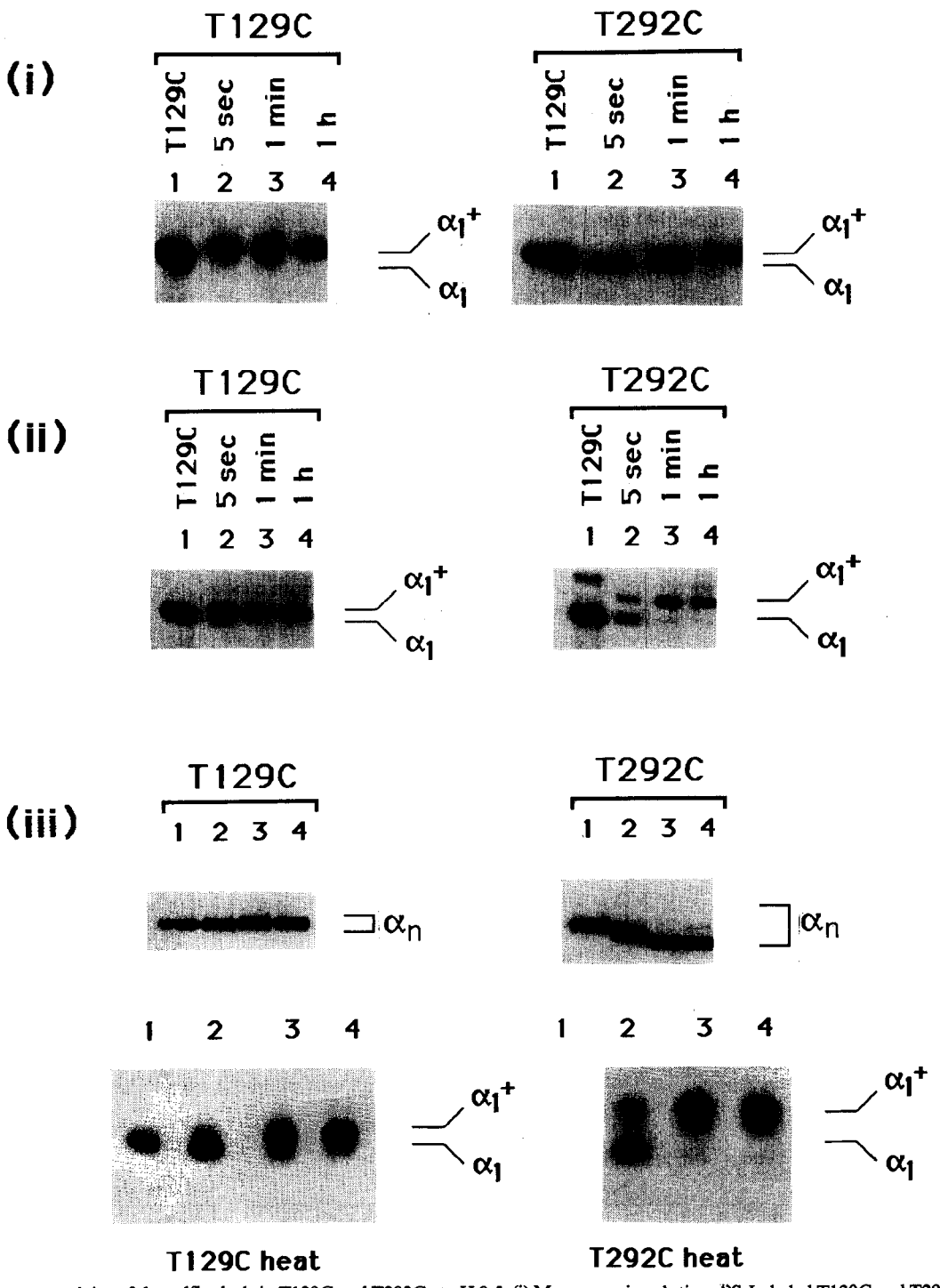

Fig. 3. Comparative reactivity of the sulfhydryls in T129C and T292C at pH 8.5. (i) Monomers in solution. ^{35}S-Labeled T129C and T292C were treated with 10 mM IASD in solution for 5 s, 1 min or 1 h. The samples were then subjected to SDS-PAGE. Autoradiograms are shown: T129C (left), T292C (right). α_1, unmodified polypeptide; α_1^+, IASD-conjugated polypeptide. (ii) Monomers on rRBC membranes. ^{35}S-Labeled T129C and T292C were allowed to assemble on rRBC for 1 h at 20°C. Washed membranes were then treated with 10 mM IASD for 5 s, 1 min or 1 h. After the addition of sample buffer and warming to 45°C, samples were subjected to SDS-PAGE. Autoradiograms of the bottom parts of the gels show monomeric αHL polypeptides: T129C (left), T292C (right). (iii) Oligomers on rRBC membranes. The parts of the gels containing oligomers (α_n) were also subjected to autoradiography: T129C (top left) and T292C (top right). The bands were then excised from the dried gels, rehydrated and heated. The gel slices and supernatants were placed in the wells of second gels and subjected once more to electrophoresis. Autoradiograms are shown: T129C (bottom left), T292C (bottom right).

3.6. *Potential extensions of the gel-shift technique*

Factors that can affect the reactivity of cysteine residues (pK_a ~9) include the extent of ionization to the reactive cysteinate anion, local electrostatic fields (IASD carries two negative charges) and steric constraints (IASD, 624 Da). The latter two difficulties can be minimized by modification with a small,

uncharged reagent, such as iodoacetamide. The latter produces no gel-shift, but we have successfully tested a two-step procedure in which ^{35}S-labeled cysteine mutants are first reacted with iodoacetamide under the desired conditions and then treated with a large excess of IASD to derivatize the remaining sulfhydryls. The extent of reaction with iodoacetamide is taken to be the fraction of the radiolabeled polypeptide that does not exhibit a gel-shift.

In the absence of a pore such as αHL, IASD is a membrane-impermeant reagent. Therefore, it could supplement treacherous techniques such as proteolytic digestion and base extraction for the topographic analysis of proteins and, for example, might find applications in investigations of the biosynthesis of membrane and secreted proteins. The high resolution of the technique with respect to primary sequence is an attractive feature. The ability to analyze polypeptides containing more than one cysteine (see section 3.5) also opens up a number of possibilities. Further, because exposed sulfhydryls react rapidly with IASD, time-resolved experiments are feasible. The time resolution could be improved by using reagents such as maleimides, which are about 1,000 times more reactive than iodoacetamides [22], combined with rapid mixing and quenching. Photochemical initiation of labeling may prove useful, as even the most reactive carbene reagents react quite selectively with sulfhydryls [23].

Acknowledgements: This study was supported by a grant from the Department of Energy. We thank Curt Wilkerson for assistance with densitometry, Lynda Zorn for help with mutagenesis and David Andrews for encouragement.

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