Mapping Disulfide Connectivity Using Backbone Ester Hydrolysis †

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Mapping Disulfide Connectivity Using Backbone Ester Hydrolysis†

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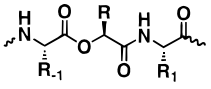
ABSTRACT: The site-specific incorporation of α-hydroxy acids into proteins using nonsense suppression can provide a powerful probe of protein structure and function. The resulting backbone ester may be selectively hydrolyzed in the presence of the peptide backbone, providing an "orthogonal" chemistry that can be useful both as an analytical tool and as a structural probe. Here we describe in detail a substantial substituent effect on this hydrolysis reaction. Consistent with mechanistic expectations, the steric bulk of the amino acid immediately N-terminal of the hydroxy acid has a large effect on the hydrolysis rate. On the basis of these results, we also describe a simple protocol for identifying disulfide loops in soluble and membrane proteins, exemplified by the α subunit of the muscle nicotinic acetylcholine receptor (nAChR). If a backbone ester is incorporated outside a disulfide loop, hydrolysis alone gives two fragments, but if the ester is incorporated within a disulfide loop, both hydrolysis and reduction are required for cleavage. This test could be useful in characterizing the disulfide topology of complex, membrane proteins.

One of the most intriguing uses of the nonsense suppression methodology for incorporating unnatural residues into proteins (*1*−*6*) is the replacement of the α-amino acids of natural proteins with α-hydroxy acids (Figure 1) (*7*, *8*). This substitution changes the normal peptide (amide) backbone to an ester, either with or without a concomitant change in side chain structure. Both in vitro and in vivo, the modification of the protein backbone has provided a useful probe of protein structure and function.

We describe here two new studies of the backbone ester methodology that significantly expand its utility. First, we demonstrate strong but understandable substituent effects on the base-induced hydrolysis of backbone esters in proteins. The results provide valuable guidelines for designing effective experiments with backbone esters. Second, we demonstrate that backbone ester hydrolysis can be developed into a decisive protocol for mapping disulfide connectivities in complex proteins. Notably, this method evaluates functional, properly folded proteins, and as such, it is in some ways less invasive than previous approaches.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes were purchased from New England Biolabs. Oligonucleotides were prepared at the Caltech Biopolymer Synthesis facility. All chemicals were purchased from Aldrich. Transcription was carried out using the T7 Magic Message Machine kit from Ambion. The dinucleotide pdCpA was synthesized as reported. Western blot analysis was carried out using the anti-hemagglutinin antibody from BabCO (catalog no. MMS-101R), the anti-mouse HRP-conjugated secondary antibody from Jackson ImmunoResearch Laboratories, and the ECL detection kit from Amersham. In vitro protein synthesis was carried out using the wheat germ extract kit from Promega.



	R
Aah	CH_3
Lah	$CH_2CH(CH_3)_2$
Vah	$CH(CH_3)_2$

FIGURE 1: Backbone structure produced when the tRNA is acylated with an α-hydroxy acid rather than with an α-amino acid.

Preparation of Mutant mRNA. The mouse muscle nAChR α-subunit containing the hemagglutinin epitope at position 347 was utilized in the experiments. All mutations were prepared using the polymerase chain reaction to generate cassettes containing the desired point mutation (either TAG or a coding triplet). Cassettes were trimmed with the appropriate restriction enzymes, purified, and ligated into the parent construct (α/pAMV-PA). Plasmid DNAs were linearized with *Not*I, and mRNA was transcribed using the Ambion T7 Magic Message Machine kit.

Preparation of α-Hydroxyacyl tRNAs. The truncated amber suppressor $tRNA_{CUA}$(-CA) was prepared by runoff transcription of *Fok*I-digested THG73-tRNA as reported previously (*6*). The α-hydroxy acids were activated as the cyanomethyl esters and coupled to the dinucleotide (pdCpA) as described previously (*17*). The acylated dinucleotides were then enzymatically ligated to the $tRNA_{CUA}$(-CA) using T4 RNA ligase as reported previously (*2*, *6*).

In Vitro Protein Synthesis. In vitro protein synthesis was carried out using the Promega wheat germ extract translation

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system according to the manufacturer's instructions. Accordingly, the following reagents were combined and allowed to stand at 25 °C for 1 h: 4.0 μL of H_2O, 1.0 μL of complete amino acids, 0.25 μL of RNAse inhibitor, 0.5 μL of tRNA (2.0 μg/μL), 0.5 μL of mRNA (1.0 μg/ μL), and 6.25 μL of lysate. After 1 h, the reaction mixtures were kept at −78 °C until further use.

In Vivo Protein Synthesis. Stage V−VI *Xenopus* oocytes were isolated as previously described and injected twice at a 24 h interval with 50 nL of a 1:1 mixture of tRNA (2.0 μg/μL) and mRNA (0.4 μg/μL at a 12:1:1:1 ratio of α, β, γ, and δ subunits) (*6*). Electrophysiological recordings were carried out ∼24 h after final injection. Macroscopic ACh-induced currents were recorded in response to bath application of 200 μM ACh at −80 mV using a two-electrode voltage clamp configuration. Bath solutions contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM Hepes (pH 7.5). Oocyte plasma membranes were isolated ∼24 h after final injection by manual stripping as reported previously (*8*). Membranes were stored at −78 °C until they were used.

Hydrolysis of in Vitro Samples. To 2.5 μL of the in vitro reaction mixture was added 2.5 μL of 4% SDS followed by 1 μL of a solution of concentrated NH_4OH in 10% SDS (1:1). Hydrolysis was allowed to proceed for minutes to hours at 25 or 95 °C as indicated in the text. Samples were then neutralized with 1 μL of a 30% glacial acetic acid solution in 10% SDS and diluted with 7 μL of H_2O followed by 14 μL of 2× SDS sample loading buffer. Samples (5 μL) were analyzed by SDS−PAGE, subjected to Western blot analysis using the anti-hemagglutinin antibody, and visualized using the ECL detection kit.

Hydrolysis and Disulfide Reduction of in Vivo Samples. Isolated plasma membranes from 25 oocytes (>10 μA whole cell current in response to 200 μM ACh per oocyte) were resuspended in 20 μL of 4% SDS and divided into 5 μL aliquots. Hydrolysis was accomplished as follows. A 5 μL aliquot was treated with 0.5 μL of a solution of concentrated NH_4OH in 10% SDS (1:1), allowed to stand for 1.5 h at 25 °C, and then neutralized with 0.5 μL of a 30% glacial acetic acid solution in 10% SDS. Disulfide reduction was accomplished as follows. An equal volume of 2× SDS sample loading buffer containing 5% β-mercaptoethanol was added to the base-treated aliquot, and the mixture was either immediately loaded or allowed to stand for 1.5 h and then loaded onto the gel. Nonreduced samples were similarly treated with an equal volume of 2× SDS sample loading buffer without β-mercaptoethanol. Samples were analyzed by SDS−PAGE, subjected to Western blot analysis using the anti-hemagglutinin antibody, and visualized using the ECL detection kit. We note that base-treated samples often appear as two bands (a lower band with the expected molecular weight and an upper band) whether the protein is synthesized in vitro (Figure 3) or in vivo (Figure 6). This doubling of the protein band is attributed to partial hydrolysis of phosphate groups on the protein, and there is some variability in this behavior.

RESULTS

Sequence Dependence of Backbone Ester Hydrolysis. Both as a diagnostic of successful suppression and as a structural probe (see below), the ability to selectively hydrolyze backbone esters is a very useful tool. Previous unpublished work from our lab indicated that this reaction was not always successful. Suspecting that local sequence context may be playing a role, we investigated ester hydrolysis across a wide range of sites within a single protein. We chose the α subunit of the mouse muscle nicotinic acetylcholine receptor (α-nAChR)[1] (*9*, *10*) in part because we had already introduced the necessary stop codon for suppression (TAG) at a number of sites in this protein (*8*, *11*). To facilitate Western blot analysis of the full-length protein, this protein contained the hemagglutinin epitope tag at position 347, which is located C-terminal to all of the sites that were tested. We have previously shown (*8*) that this mutation does not detectably alter the function of the intact receptor. Three α-hydroxy acids were studied: the analogues of Ala, Leu, and Val, termed Aah, Lah, and Vah, respectively (ah being α-hydroxy). Backbone esters were incorporated at 29 different sites via suppression of the TAG codon by exogenously introduced hydroxyacyl $tRNA_{CUA}$ (Table 1). For most studies, the suppression was studied with an in vitro, wheat germ extract translation system. Several studies were also performed with the in vivo *Xenopus* oocyte system. All studies used our previously described suppressor tRNA THG73 (*12*), which has been optimized for the oocyte system. It is not known whether THG73 is optimal for the wheat germ extract system.

The results of the in vitro studies are summarized in Table 1. While suppression efficiency is not the major focus of this work, we can make some general observations about the present system. Often, quite substantial quantities of ester-containing protein can be obtained by suppression. The highest levels (+++) of suppression (TAG codon, hydroxyacyl tRNA) produce roughly as much protein as translation of the wild-type protein without nonsense suppression (standard triplet codons, endogenous aminoacyl tRNAs). In >95% of the experiments, the levels of suppressed protein obtained were within 1 order of magnitude (++ and +++, Table 1) of the levels of the wild-type protein. Poor suppression (+, Table 1) was observed in only 5% of the experiments. In addition, it appears that suppression with Vah is superior to that with Aah. Poor suppression (+) was observed only when Aah was used. Further, side by side comparison of Vah and Aah suppression efficiencies at the 29 sites that were tested reveals that Vah outperforms Aah ∼50% of the time and is outperformed by Aah only ∼24% of the time. While these comparisons are qualitative, the trends seem nonetheless clear.

The efficiency of hydrolysis using aqueous NH_4OH was rated −, +, ++, or +++ on the basis of the relative intensities of the full-length and the cleavage fragment band from base-treated samples analyzed using SDS−PAGE followed by Western blotting. Figure 2 shows representative results. Again, while this rating system is a qualitative one, patterns in the data are evident.

The time course of cleavage for a +++ hydrolysis site was studied for Vah at position 253. As shown in Figure 3A, hydrolysis of the Vah backbone ester is essentially complete after 30 min at 25 °C, and requires only 2 min at

[1] Abbreviations: nAChR, nicotinic acetylcholine receptor; Vah, α-hydroxy analogoue of Val; Lah, α-hydroxy analogoue of Leu; Aah, α-hydroxy analogoue of Ala.

Table 1: Summary of the Results of Backbone Ester Incorporation and Hydrolysis[a]

TAG Mutation[b]	Sequence[c]	Suppression[d]			Hydrolysis[e]		
		Aah	Lah	Vah	Aah	Lah	Vah
122	PP-A-IF	++		+++	+		++
123	PA-I-FK	+++		++	+++		+++
124	AI-F-KS	++		+++	+		+
127	KS-Y-CE	++		++	+		+
131	EI-I-VT	+++		++	–		–
132	II-V-TH	+++		+++	–		–
132[f]	IS-V-TH	++		+++	++		+++
134	VT-H-FP	+		++	–		++
144	CS-M-KL	+++		++	++		+++
145	SM-K-LG	++		++	+++		+++
146	MK-L-GT	+++	+++	++	++	++	+++
193	SC-C-PT	+++	++	+++	++	++	++
194	CC-P-TT	+++		++	++		+++
206	HF-V-MQ	++		+++	++		++
211	RL-P-CL	++	++	+++	++	++	++
221	II-P-CL	++		++	–		–
236	YL-P-TD	++	+++	+++	++	++	++
240	DS-G-EK	++		+++	++		+++
243	IK-M-TL	+++		++	++		++
244	KM-T-LS	+	+++	+++	+	++	++
245	MT-L-SI	+	+++	++	+	++	++
247	LS-I-SV	++		++	+++		+++
250	SV-L-LS	++		++	–		+
251	VL-L-SL	+++		++	++		++
252	LL-S-LT	++		++	++		+
253	LS-L-TV	++	+++	+++	++	+++	+++
255	LT-V-FL	++	+++	+++	–	++	++
257	VF-L-LV	++		++	++		++
258	FL-L-VI	++		+++	++		++

[a] Proteins were synthesized in vitro, and hydrolyses were carried out for 1.5 h at 25 °C. [b] Location of ester incorporation into the mouse muscle nAChR α subunit. Positions within the protein, using the conventional assignment, are as follows. Residues below and including position 206 are in the N-terminal, extracellular domain. Residues 211, 221, and 236 are in the first transmembrane domain (M1). Residue 240 is in the intracellular loop between M1 and M2. Residues 243−258 are in M2. [c] Local sequence near the site of mutation. The middle residue of the five is replaced with the ester (i.e., it is the site designated in the previous column). [d] Suppression efficiency with the noted hydroxy acid. Efficiency is noted as + for poor, ++ for good, and +++ for very good. [e] Hydrolysis efficiency using the criteria defined in the legend of Figure 2. [f] For this experiment, the TAG is at position 132, and the (conventional) I131S mutation has also been introduced. See the text.

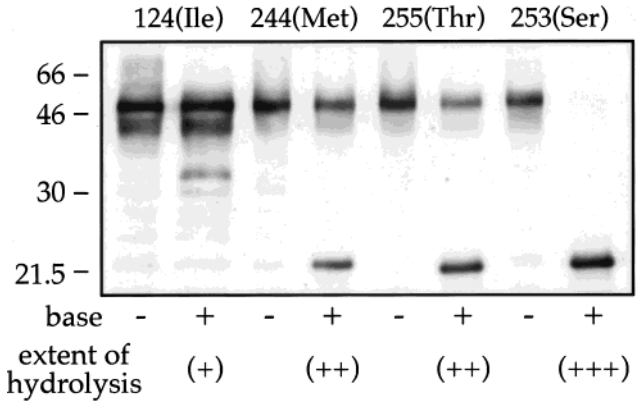

FIGURE 2: Western blot analysis of hydrolysis reactions, showing representative examples for each class of hydrolysis efficiency. To identify lanes, the number indicates the site at which the hydroxy acid (always Vah) was incorporated, and the amino acid in parentheses identifies the $i - 1$ residue. Proteins were synthesized in vitro, and hydrolyses were carried out for 1.5 h at 25 °C. Note that in this, and all subsequent gels, only one cleavage band is seen because the epitope tag, which is located C-terminal of all cleavage sites that were probed, is present in only one fragment. For example, the band from cleavage at position 124 is at a higher molecular weight than the others because the cleavage occurs nearer the N terminus, leaving a larger C-terminal fragment to be visualized. The 124(Ile) lanes were loaded with 2.5 times as much protein as the other lanes.

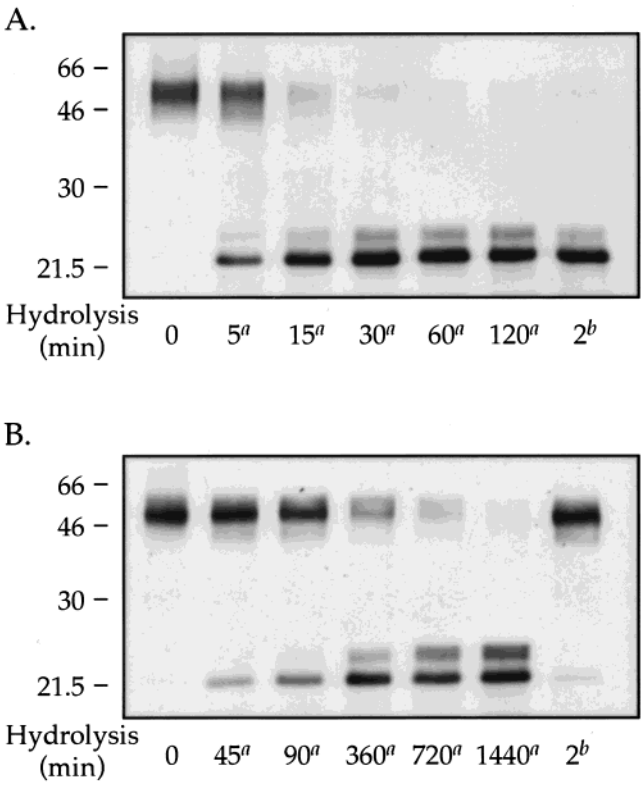

FIGURE 3: Time course for hydrolysis of (A) an efficient site (αLeu253Vah) and (B) a recalcitrant site (αLeu252Vah). Proteins were synthesized in vitro, and hydrolyses were carried out for the indicated periods of time at (a) 25 or (b) 95 °C.

95 °C. An analogous study of a recalcitrant + site, Vah at position 252, is shown in Figure 3B. At this site, hydrolysis is complete after 24 h at 25 °C, but 2 min at 95 °C has little or no effect.

Consideration of all the data, a total of 66 hydrolysis experiments (29 sites with Aah, 29 sites with Vah, and 8

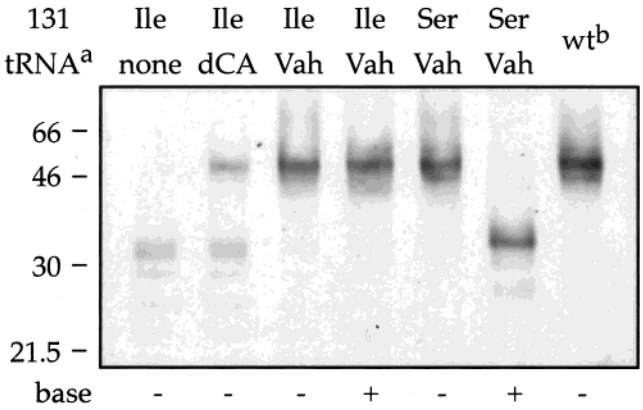

FIGURE 4: Rational design of an efficient hydrolysis site. The final lane is the wild-type protein. For all other lanes, the TAG stop codon is at position 132, the residue at position 131 (Ile or Ser) is listed, and the identity of the suppressor tRNA included in the translation mixture is also listed. (a) tRNA key. None implies no suppressor tRNA added; dCA is the full-length suppressor tRNA but with no amino acid or hydroxy acid appended, and Vah means that the hydroxy acid Vah is appended to the tRNA. The none and dCA lanes are controls demonstrating that little or no full-length protein is produced under these conditions. Proteins were synthesized in vitro, and hydrolyses were carried out for 1.5 h at 25 °C.

sites with Lah; see Table 1), reveals some definite trends. The global conclusion is that the side chain identity of the $i - 1$ amino acid (Figure 1) is most crucial. Ester hydrolysis fails in 9 of 66 hydrolysis attempts. In each case, the $i - 1$ residue is a β-branched amino acid (I six times, T two times, and V one time). We propose that the steric bulk of these side chains retards hydrolysis. As shown in Figure 1, the $i - 1$ side chain is closest to the ester carbonyl that will be attacked by base, and so the $i - 1$ residue is expected to have the largest steric effect. Two other hydrolysis experiments with $i - 1$ being I and one other hydrolysis experiment with $i - 1$ being V are in the + category, but none are ++ or +++. Interestingly, hydrolysis with T at the $i - 1$ site is more variable; only one T experiment is − and another T experiment is +, but five experiments are ++. While T is β-branched, it is also more polar than I or V and may, therefore, be less of a hindrance to attack by base.

There are also nine examples of + hydrolysis levels; five include β-branched residues at the $i - 1$ site, and one has P at $i - 1$, another potentially sterically demanding residue. The remaining + sites (positions 127 and 244) have S and M, respectively, at the $i - 1$ site. Interestingly, for both of these sites, the $i - 2$ residue is K. Additional work would be required to determine if this apparent hindrance by a lysine at the $i - 2$ position is just a coincidence.

To further test the steric bulk hypothesis, a construct was prepared with TAG at position 132, and the natural I at position 131 mutated to S. This was anticipated to transform an inactive site (I at $i - 1$) to an active site (S at $i - 1$; see below). Indeed, as shown in Table 1 and Figure 4, the I131S mutation "rescues" hydrolysis when an α-hydroxy acid is incorporated at position 132. This strongly supports the dominant influence of the $i - 1$ residue in determining hydrolysis efficiency.

With respect to residues that are favorable for hydrolysis, C, K, and A at the $i - 1$ site always allow efficient (++ or +++) hydrolysis, as does S with the exception of the previously discussed position 127 site. Generally, small polar

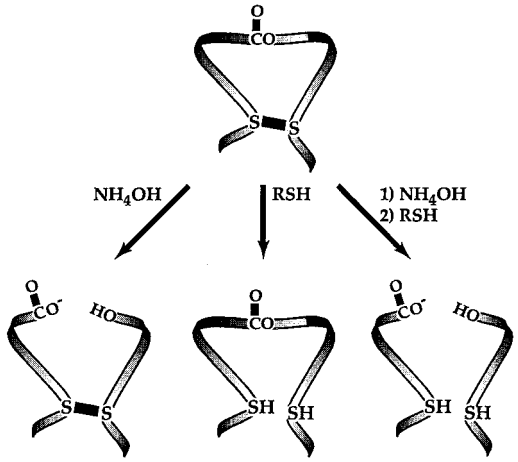

FIGURE 5: Schematic illustrating the use of backbone ester hydrolysis in identifying sites within disulfide loops.

residues seem best. Supporting this, large, hydrophobic (but not β-branched) residues such as L and F never give +++ hydrolysis efficiencies.

Perhaps surprisingly, the bulkier Vah residue is, on balance, superior to Aah for hydrolysis efficiency. This is not a large effect, but is one that is worth considering when designing experiments.

Ester Hydrolysis as a Probe of Disulfide Topology. The ester hydrolysis conditions developed here represent an "orthogonal" chemistry, a reaction that operates on the specifically introduced ester group and not on any of the intrinsic functionality of the protein. This allows the development of novel chemical probes of structure. Here we provide a proof of concept for the use of backbone esters in mapping disulfide topology.

As illustrated in Figure 5, if a backbone ester is incorporated into a disulfide loop, then different chemical treatments produce distinct bands in gel electrophoresis. If the protein is exposed only to hydrolysis conditions (NH_4OH) or only to reducing conditions (RSH), one still produces a full-length protein. However, exposure of the protein to both hydrolysis and reduction produces two fragments. This provides a clear distinction between a residue that is not within a loop (hydrolysis alone gives two fragments) and one that is within a loop (both hydrolysis and reduction required for cleavage).

To develop this protocol, we used a site within the "signature" disulfide loop of the nAChR formed between Cys128 and Cys142 (*9, 10*). Note that there was some initial concern about the true orthogonality of the two reactions. One could envision that the harsh conditions of the NH_4OH treatment could disrupt a disulfide by a nucleophilic displacement (S_N2) mechanism. Similarly, thiolate in the reducing medium could attack an ester carbonyl, resulting in cleavage. The conditions described here avoid these complications.

We incorporated Vah at position 132 of an α-nAChR that also contained the I131S mutation (Table 1). For this study, the protein was expressed in vivo (*Xenopus* oocytes), and the β, γ, and δ nAChR subunits were coexpresssed with the mutant α subunit, producing intact functional receptors on the surface of the oocyte. In this way, we could be certain that the 128−142 disulfide was properly formed, a result that could not be guaranteed with the in vitro translation system. Surface receptors were isolated and subjected to the conditions described in Figure 5.

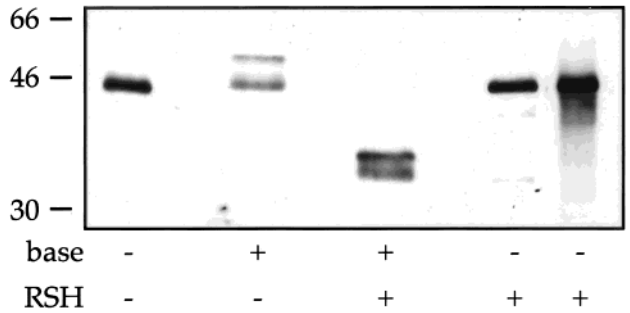

FIGURE 6: Cleavage of a site within a disulfide loop, as diagramed in Figure 5. The protein that was studied had Vah incorporated at position 132, and the Ile131Ser mutation (Figure 4). For all but the rightmost lane, proteins were synthesized in vivo, and hydrolyses were carried out for 1.5 h at 25 °C. Treatment with β-mercaptoethanol (RSH) simply indicates whether the reagent was included in the SDS−PAGE sample loading buffer. The rightmost lane is from an in vitro translation, conditions under which the 128−142 disulfide is presumably not formed (Table 1 and Figure 4). Treatment with β-mercaptoethanol gives only the full-length protein.

The results, as shown in Figure 6, indicate that conditions can be found that clearly give the expected results. Since the α132Vah ester is located within a disulfide loop, base hydrolysis under our standard conditions (1.5 h at 25 °C) does not fragment the protein, while hydrolysis followed by reduction provides complete cleavage. In the RSH only lane (lane 4), a faint band appears at approximately the molecular weight of the expected cleavage fragment. In another experiment with the same samples described in the legend of Figure 6, prolonged treatment (1.5 h) with β-mercaptoethanol did not increase the intensity of this band. In addition, the same band does not appear in an analogous in vitro sample (Figure 6). Thus, this additional band is an artifact of the in vivo translation system. The aggregate results of Figure 6 clearly confirm that position α132 is located within a disulfide loop, and establish the viability of the protocol.

DISCUSSION

Incorporation of backbone esters is one of the most interesting applications of the nonsense suppression methodology, and here we show that it is potentially a very useful tool. We recognize that our sampling of sites is neither exhaustive nor unbiased. We took advantage of the many TAG sites we had previously introduced into the α subunit of the nAChR in our earlier structure−function studies. Nonetheless, sites from both the extracellular ("soluble") and transmembrane regions of the receptor are equally represented, and we feel the data are adequate for establishing the key trends.

Backbone ester incorporation by nonsense suppression is generally quite efficient. Efficient incorporation (++ or +++) is always observed with Vah and Lah, while Aah occasionally gives poor results. The preference for the bulkier, more hydrophobic residues (Vah and Lah) could reflect some feature of the translation machinery. Alternatively, the hydroxyacyl tRNA may be more stable with Vah and Lah, perhaps because the steric bulk of the side chain retards hydrolysis of the 2′−3′ linkage to the ribose.

A clear conclusion from the study presented here is that the most important factor in determining hydrolysis ef-

ficiency is the steric bulk of the side chain of the amino acid in the $i - 1$ position. The β-branched residues I and V universally retard hydrolysis. The β-branched residue T gives mixed results, although never +++, suggesting that hydrophobicity may also be a factor. This trend is mechanistically sensible. The $i - 1$ side chain is nearest the ester carbonyl that is attacked by the base in the hydrolysis reaction, and numerous classical physical organic studies have shown that steric effects of the sort seen here can have a substantial influence on the rates of ester hydrolysis (*13*).

Compelling evidence that the $i - 1$ side chain determines hydrolysis efficiency comes from the rational conversion of a hydrolysis resistant − site to a readily hydrolyzed +++ site. While α132, with the sequence I-Xah-T, cannot be hydrolyzed, mutation of this sequence to S-Xah-T renders the ester easily hydrolyzed.

To favor hydrolysis, it appears that small, hydrophilic residues are best, although acceptable results (++) are seen with many side chains. Nevertheless, to fully optimize ester hydrolysis, we recommend having S, C, K, or A and avoiding I, V, and T at the $i - 1$ site. At an appropriate site, backbone ester hydrolysis can be predictably and reliably achieved.

Previous studies of backbone ester hydrolysis in our laboratories gave mixed results. In many of our early studies, we were unable to detect complete hydrolysis. We now appreciate that this occurred because we were focused on a transmembrane region of the receptor that was rich in bulky, hydrophobic residues such as I and V. Many sites we studied had I or V at the $i - 1$ site, retarding hydrolysis.

Another trend is the small but clear bias for Vah esters to be more easily hydrolyzed than Aah esters. Perhaps a more bulky ester side chain (R in Figure 1) causes a twisting around the ester C−O bond into a conformation that is more favorable for hydrolysis. When coupled with the greater suppression efficiency, Vah may well be the α-hydroxy acid of choice.

Backbone ester incorporation also allows some quantitative evaluation of the nonsense suppression methodology. For samples originating from in vivo suppression experiments (Figure 6 and ref *8*), ester hydrolysis appears to be quantitative within the detection limits of the system. This attests to the fidelity of the in vivo nonsense suppression method. That is, the lack of a residual, nonhydrolyzable, full-length protein band following base treatment suggests that read-through of the stop codon by endogenous aminoacyl tRNAs or editing and reacylation of the suppressor tRNA does not occur to any measurable extent. The efficiency of incorporation of α-hydroxy acids is also supported by the in vitro experiments. Inclusion in the translation reaction of the uncharged suppressor tRNA (termed "dCA"), in place of hydroxyacylated tRNA, results in the synthesis of some full-length protein, indicating that endogenous synthetases will charge the suppressor tRNA (Figure 4, dCA lane). This charging would of course involve acylation with amino acids and would therefore produce a full-length protein resistant to our hydrolysis conditions. However, inclusion of the suppressor tRNA charged with an α-hydroxy acid results in a full-length protein that can be completely hydrolyzed (Figure 4, 131Ser132Vah lane). Thus, it appears that hydroxyacylated tRNAs are not converted to aminoacylated tRNAs to any measurable extent in vitro.

With a reliable approach to ester hydrolysis in hand, we have developed a protocol in which ester hydrolysis is used to establish whether a residue is in a disulfide loop. We believe this could be quite useful in the study of the integral membrane proteins because (1) high-resolution structural information is sparse and (2) their extracellular domains are in an oxidizing environment and would be expected to have disulfide bonds. For example, the P2X and ENaC ion channels represent two recently discovered, physiologically interesting structures (*14*). Both contain two transmembrane domains separated by a large, extracellular domain that contains 10 cysteines, which are no doubt disulfide-linked to some extent. The strategy of Figure 5 provides a way of probing the topology of such a disulfide network. This approach considers only fully functional channels with all disulfides intact, and it involves a mutation (amide-to-ester) that is among the most subtle imaginable. As such, we feel it is significantly less invasive than other approaches (*15*) which generally introduce substantial mutations such as protease cleavage sites or that mutate away cysteines, and thus evaluate compromised channels.

As shown in Figure 6, the strategy of Figure 5 is quite simple to implement. The pattern of Figure 6 unambiguously places the site of ester incorporation within a disulfide loop. Of course, a different pattern is seen for an ester not in a loop, as in the many examples listed in Table 1. The results from this approach are unambiguous if only a single disulfide is being evaluated, as with the nAChR. In systems such as the P2X and ENaC ion channels discussed above, with many Cys residues, and thus many possible disulfide topologies, a complete assignment may not always be possible. The simplest case (which is fairly common) is one in which disulfides are formed by Cys residues that are consecutive in sequence, and our approach gives unambiguous results if esters are systematically inserted between each pair of Cys residues. More complicated topologies would require the use of either multiple ester substitutions or a combination of ester incorporation and conventional mutagenesis to arrive at an unambiguous assignment.

In summary, the high efficiency and broad scope of α-hydroxy acid incorporation (*7*, *8*), coupled with the increased stability of the hydroxyacyl tRNA (vs the aminoacyl tRNA used to incorporate amino acids) (*16*) and the much simpler synthesis of hydroxyacyl tRNAs (no protecting group required) (*17*), suggest a prominent role for hydroxy acids in future suppression studies. The disulfide mapping strategy presented here represents one potentially powerful application. We also anticipate that in future studies involving incorporation of biophysical probes or reactive functionalities into proteins, better success may be achieved with an α-hydroxy acid than with the corresponding α-amino acid.

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