Site-Specific Protein Modification Using a Ketone Handle

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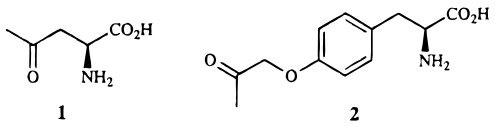
The reactive thiol side chain of the natural amino acid cysteine has been widely exploited for the selective modification of proteins with a variety of biophysical probes,[1] as a structural probe,[2] to stabilize proteins and control enzyme activity,[3] and for the generation of semisynthetic proteins.[4] The availability of a second, nonproteinogenic amino acid with such selective reactivity would be very useful in situations requiring the incorporation of two different labels into a protein, selective derivatization of unpurified protein, intracellular cross-linking or protein modification, or where a unique cysteine residue cannot be engineered into a protein. Although biosynthetic approaches involving chemically-modified or synthetic aminoacyl-tRNAs can be used to incorporate probes directly into proteins, the biosynthetic machinery places constraints on the nature of the side-chain functionality which can be incorporated.[5] Here we report a new strategy for labeling proteins site-specifically which relies on an unnatural amino acid with an electrophilic ketone side chain (Scheme 1). The unnatural keto amino acid is introduced into a unique site in a protein using the method of unnatural amino acid mutagenesis.[6] The carbonyl group of this amino acid can then be modified with a broad range of molecules containing hydrazide or alkoxyamine groups. To demonstrate this approach, T4 lysozyme containing an unnatural keto amino acid was prepared and then reacted with a fluorescein hydrazide to provide T4 lysozyme labeled site-specifically with fluorescein via a stable hydrazone linkage.

The keto group is an attractive "handle".[7] It is sufficiently stable to be incorporated into a protein in an unprotected form using a crude *Escherichia coli in vitro* extract; and yet it will react rapidly with hydrazides and alkoxyamines in aqueous solution to form hydrazones and oximes, respectively.[8] Furthermore, both hydrazones and oximes are stable under physiological conditions. The reaction of a ketone with a hydrazide is orthogonal to the functional groups present in proteins.[9] This chemistry has been used to label peptides and proteins at their *N*-termini by oxidizing an *N*-terminal serine with periodate to the corresponding aldehyde which is subsequently coupled to an appropriate hydrazide.[8a] In addition, peptide dendrimers and synthetic proteins have been constructed by coupling peptide fragments via hydrazone or oxime linkages.[8b−d]

Initially, we considered two different keto amino acids, **1** and **2**. These two amino acids differ both in the proximity of the carbonyl group to the protein backbone and in their reactivity due to the electron-withdrawing α-phenoxy group and differential steric effects. The longer homologues of **1** were not employed because they are prone to cyclic imine formation.[10] The required *N*-protected amino acid of ketone **1** was prepared from 4,5-dehydroleucine by protection of the α-amine with nitroveratryl chloroformate followed by ozonolysis of the terminal olefin. Ketone **2** was prepared similarly from *O*-(methylallyl)tyrosine, which was synthesized by treating the copper(II) complex of tyrosine with methylallyl bromide in alkaline solution.[11]



1 **2**

The efficiency with which **1** and **2** could be incorporated into a protein by the biosynthetic machinery was tested at two solvent accessible sites (Ser^{44} and Ala^{82}) in the protein T4 lysozyme (T4L) using unnatural amino acid mutagenesis.[6] An amber suppressor tRNA was charged with the keto amino acids **1** and **2** using established methods, and the aminoacylated suppressor tRNAs were combined with mutant T4L genes encoding amber codons at site 44 or site 82 in an *E. coli in vitro* transcription/translation extract.[12] Protein production levels were judged by catalytic activity and by SDS-PAGE and autoradiography of the soluble fractions of transcription/translation reactions charged with ^{35}S-methionine.[13] At both sites 44 and 82 ketone **1** is incorporated with only 5% efficiency (consistent with the size and polarity of the side chain),[5a] while ketone **2** is incorporated with 30% efficiency.[14] Further tests established that ketone **2** is incorporated with 20−30% efficiency at sites in several different proteins, including aspartate transcarbamylase, keto-

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(5) (a) Cornish, V. W.; Benson, D. R.; Altenbach, C. A.; Hideg, K.; Hubbell, W. L.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2910−2914. (b) For example, three glycosyl amino acids (α-*O*-rhamnosyl serine, N^{ϵ}-[β-*C*-carbonyl(*N*-acetylglucosamine)]lysine, and N^{β}-[β-*C*-carbonyl(*N*-acetylglucosamine)]-2,3-diaminopropanoic acid) were synthesized as the necessary aminoacyl-pdCpAs and tested for incorporation at sites 44 and 82 in T4L using unnatural amino acid mutagenesis. All three were incorporated with <1% efficiency: Cornish, V. W.; Michael, K.; Hoffmann, M.; Kessler, H.; Schultz, P. G. Unpublished results.

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(14) The incorporation efficiency is defined as the ratio of the amount of T4L produced by suppressing a gene containing an amber codon *in vitro* with a given aminoacyl-$tRNA_{CUA}$ relative to the amount produced from the wt gene *in vitro*. As a control, the amount of T4L produced when the aminoacyl-tRNA is replaced by an unacylated tRNA is shown to be ≤1%.

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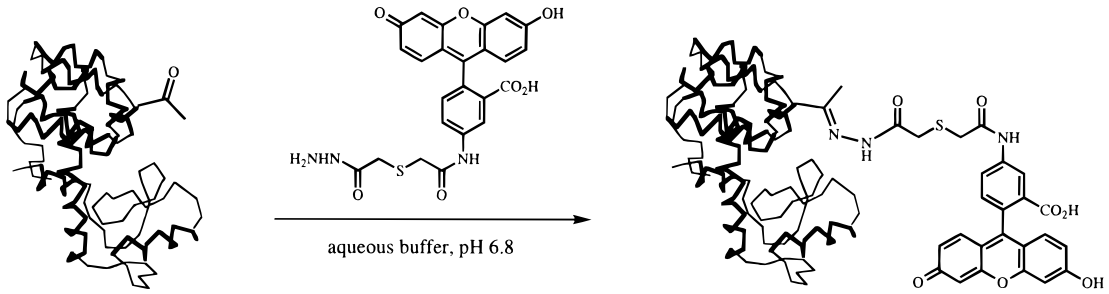
Scheme 1



steroid isomerase, and thymidylate synthase.[15] Based on these results, ketone **2** was chosen for use in the subsequent chemical derivatization experiments.

In order to determine the efficiency with which proteins containing ketone **2** can be modified, a T4L mutant containing **2** at site 82 was isolated from a large-scale *in vitro* reaction and labeled with a commercially available fluorescein hydrazide (Scheme 1). As a control, wild-type (wt) T4L was isolated from a large-scale *in vitro* reaction and taken through identical labeling conditions.[16] T4L Ala^{82} → **2** and wt T4L were prepared from 10 mL and 3 mL *in vitro* reactions, respectively, and partially purified by ion-exchange chromatography using tandem DEAE-CM cartridges to yield ca. 10 μg of each protein. Portions of these crude protein solutions were exchanged into 100 mM potassium phosphate, pH 6.8, 0.5 M sodium chloride and concentrated. Fluorescein hydrazide was added to the protein solutions such that the labeling reactions contained 1 μM T4L, 1 mM fluorescein hydrazide, 20% dimethyl sulfoxide, and 80 mM potassium phosphate, pH 6.8, 0.4 M sodium chloride. The mixture was allowed to react for 36 h at room temperature and then was desalted and purified to homogeneity by ion-exchange chromatography. A comparison of the fluorescence spectra of labeled T4L Ala^{82} → **2** and "labeled" wt T4L shows that only the protein containing the ketone at site 82 has been labeled with fluorescein (Figure 1). Based on a comparison to the fluorescence emission spectrum of T4L labeled nonspecifically with the NHS ester of fluorescein, the extent of labeling is judged to be approximately 50%.[17] This strategy should facilitate the versatile alteration of protein structure, including controlled post-translational modification with polysaccharides and terpenes and the introduction of biophysical probes. Currently the keto "handle" is being used for the site-specific introduction of novel fluorophores[18] into proteins to produce biosensors which can report specific protein activities within individual, living cells.[1d]

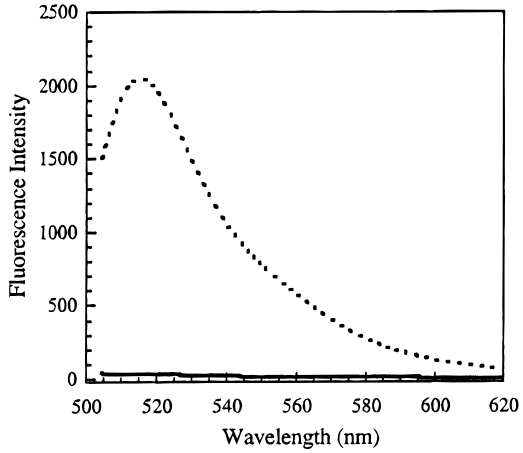


Figure 1. Fluorescence emission spectra (uncorrected) of wild-type T4 lysozyme (solid line) and of the T4 lysozyme mutant Ala^{82} → ketone **2** (dotted line) that have been treated with fluorescein hydrazide; excitation at 490 nm.

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Supporting Information Available: Experimental procedures for the syntheses of ketones **1** and **2**, for protein purification, for the labeling reactions, and for the fluorescence experiments (7 pages). See any current masthead page for ordering and Internet access instructions.

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(16) For both the T4L mutant Ala^{82} → ketone **2** and wt T4L, the protein purification, labeling, and fluorescence experiments were also done at pH 4.5, giving the same results.

(17) *In vivo* produced T4L was labeled non-specifically with the NHS ester of fluorescein. The extent of labeling was judged to be approximately 1:1 dye to protein based on the ratio of A_{490} to A_{280}, and the concentration of fluorescein was determined from the A_{490}. A caveat to this quantification method is that the fluorescence of fluorescein is influenced by the exact protein environment.

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