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Chemistry and Photochemistry Attending the Inactivation of *Escherichia coli* β-Hydroxydecanoyl Thiol Ester Dehydrase by an Acetylenic Diazoketone

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Abstract: β-Hydroxydecanoyl thiol ester dehydrase from *Escherichia coli*, an enzyme that catalyzes both dehydration and allylic rearrangement reactions, has been shown previously to undergo mechanism-based inactivation by the acetylenic substrate analog 1-diazo-4-undecyn-2-one (DUO). Details of the chemistry and photochemistry of DUO are now presented. Analysis of DUO-inactivated dehydrase by ^{15}N NMR spectroscopy indicates that DUO quantitatively alkylates histidine-70. Long-wavelength photoirradiation leads to spectrophotometrically observable changes in the DUO–dehydrase adduct. The structural changes were characterized by a combination of methods. Samples of protein that had been inactivated with [1-^{13}C]-, [2-^{13}C]-, and [3-^{13}C]DUO were analyzed by ^{13}C NMR spectroscopy, both prior and subsequent to photoirradiation. By comparisons of the chemical shifts of the enriched atoms of the inactivator moiety with those of model compounds, it was confirmed that the α-diazoketone moiety is retained in DUO-inactivated dehydrase and that photoirradiation leads to Wolff rearrangement of the α-diazoketone moiety followed by attack of a nucleophile on the resulting ketene. Proteolytic degradation of photoirradiated, DUO-inactivated dehydrase gave peptides that were analyzed by Edman sequencing and by mass spectrometry. The results are consistent with a single protein modification (at H70), and attack on the ketene by water, leading to a carboxylic acid. Samples of intact native, DUO-inactivated, and photoirradiated, DUO-inactivated dehydrase were analyzed by electrospray ionization mass spectrometry, the results of which clearly support the hypothesis that the ketene suffers attack by water. In light of recent results from X-ray crystallography, it is proposed that the water that attacks the ketene is one of two bound water molecules with specific roles in the binding and/or catalytic turnover of substrate. A Michael addition–elimination mechanism is presented to explain the unexpected hydrolytic lability of the DUO–dehydrase adduct.

The key enzyme in the biosynthesis of monounsaturated fatty acids in *Escherichia coli*, β-hydroxydecanoyl thiol ester dehydrase, mediates the dehydration of (*R*)-3-hydroxydecanoyl-ACP (**1**; R = acyl carrier protein; ACP) to (*E*)-2-decanoyl-ACP (**2**; R = ACP) and the allylic rearrangement[1] of **2** to (*Z*)-3-decenoyl-ACP (**3**; R = ACP). Compound **2** undergoes reduction to the saturated thiol ester, which is elongated to full-length saturated fatty acids, while **3** is elongated without prior reduction, leading to monounsaturated fatty acids including (*Z*)-9-hexadecenoic acid and (*Z*)-11-octadecenoic acid (see Scheme 1).

Extensive studies in Bloch's laboratory[2,3] and in ours[1,4,5] have

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(1) Schwab, J. M.; Henderson, B. S. *Chem. Rev.* **1990**, *90*, 1203–1245.
(2) Helmkamp, G. M., Jr.; Bloch, K. *J. Biol. Chem.* **1969**, *244*, 6014–6022.
(3) Bloch, K. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. 5, pp 441–464.
(4) Schwab, J. M.; Ho, C.-K.; Li, W.-b.; Townsend, C. A.; Salituro, G. M. *J. Am. Chem. Soc.* **1986**, *108*, 5309–5316.
(5) Annand, R. R.; Davisson, V. J.; Kozlowski, J. F.; Schwab, J. M. *J. Am. Chem. Soc.* **1993**, *115*, 1088–1094.

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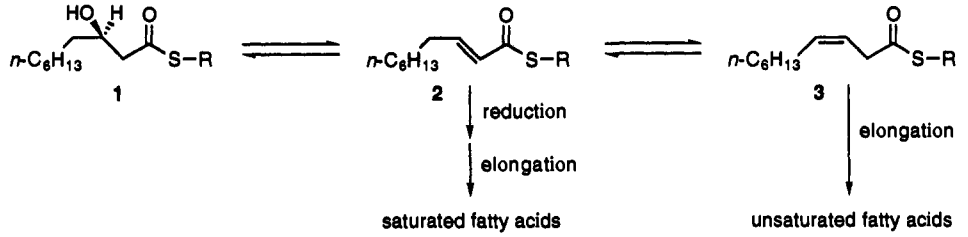
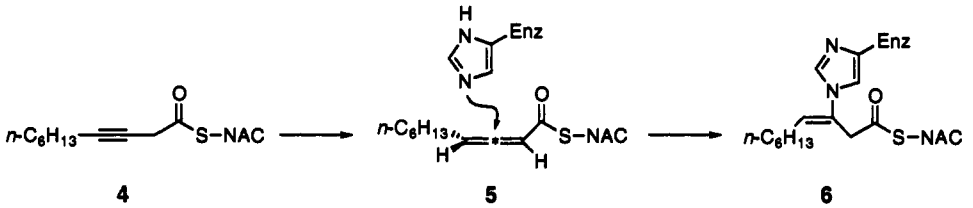
Scheme 1



Scheme 2



implicated a histidine residue as the sole active site base that mediates deprotonation of the substrates and (as its conjugate acid) protonation of reaction intermediates. Perhaps the most convincing evidence in support of histidine's role in catalysis comes from the irreversible inactivation of dehydrase by the mechanism-based inactivator 3-decynoyl-NAC (**4**; NAC = *N*-acetylcysteamine). Dehydrase isomerizes **4** to allene **5**,[2,4,6] which rapidly alkylates a histidine residue,[4] subsequently shown to be H70.[7] (See Scheme 2.) While chemical modification[2] and sequence alignment[8] have suggested that a tyrosine or an aspartic acid residue, respectively, might also play a role in catalysis, these claims are to date purely speculative.

1-Diazo-4-undecyn-2-one (DUO) was designed as a cross-linker of dehydrase's active site amino acids.[9] The rationale was that as an analog of thiol ester **4**, DUO would alkylate H70, and once the inactivator had been tethered at the active site, photoirradiation would lead to the loss of N_2 and the generation of either α-ketocarbene **9** or ketene **8** (via Wolff rearrangement). Either one of these species would be sufficiently reactive to modify a second active site amino acid and in so doing cross-link the protein. (See Scheme 3.) We now present a detailed characterization of the modifications to both the protein and the inactivator that result from inactivation of dehydrase by DUO and from photoirradiation of the initial DUO–dehydrase adduct.

Results

In a preliminary communication we reported that DUO is indeed a mechanism-based inactivator of dehydrase.[9] It was presumed that DUO alkylates H70 (cf. **7**), and confirmation initially was sought by amino acid analysis of DUO-inactivated dehydrase. The results (Table 1) were inconclusive albeit indicative. Although the histidine content of DUO-inactivated dehydrase was reduced from that of native, wild-type dehydrase (which has two histidines per subunit[2,7]), it was reduced by only one-third of a residue per subunit. No other significant DUO-dependent changes in the amino acid composition were observed. Following photoirradiation of DUO-inactivated dehydrase (see below), slightly over one unmodified histidine was found per subunit. As a positive control, the histidine content of dehydrase inactivated by 3-decynoyl-NAC (which is known to modify exactly one histidine per subunit[4,10]) was also found to be a little more than one per subunit. This value is essentially identical to that which was reported originally by Helmkamp and Bloch.[2]

Definitive evidence for the specific modification by DUO of one histidine per subunit came from ^{15}N NMR analysis of DUO-inactivated [U-^{15}N]dehydrase (Figure 1). As we have described elsewhere,[5] the ^{15}N NMR spectrum of [U-^{15}N]dehydrase (spectrum A) exhibits four imidazole nitrogen signals, two each for H70 and H129. The signal assignments for H70 come from the analysis of protein made from [$^{15}N^{\delta 1}$]histidine. Inactivation of dehydrase by 3-decynoyl-NAC (spectrum B) leads to the alkylation of $N^{\epsilon 2}$ of H70 (cf. **6**), which now exhibits a typical type-α chemical shift of −187.2 ppm. $N^{\delta 1}$ of H70 is type-β in the inactivated enzyme, with a chemical shift of −103.8. For reasons that are not clearly understood,[5] the signal for the type-α nitrogen of H129 is broadened in spectrum B. On the basis of the similarity of ^{15}N NMR spectra B and C in Figure 1, it can be concluded that inactivation of dehydrase by DUO or 3-decynoyl-NAC leads to nearly identical modifications of H70 (cf. structures **6** and **7**). The fact that there is no trace in spectrum C of a signal at −127.5 ppm, corresponding to $N^{\epsilon 2}$ of H70 of the native protein,[5] indicates that the modification of H70 by DUO is quantitative. This finding correlates well with the results of stoichiometry experiments using radiolabeled DUO.[9]

α-Diazoketones typically exhibit a broad, very low-intensity absorption at ca. 400 nm.[11] Accordingly, DUO-inactivated dehydrase was photoirradiated for ca. 8 h using a medium-pressure Hg lamp equipped with a uranium yellow filter. These conditions were chosen in order to eliminate wavelengths below 330 nm and thus to minimize damage to the protein.[12,13] The photoirradiation- and time-dependent alteration of the diazoketone chromophore could be followed spectroscopically, as illustrated in Figure 2. As a control, a sample of native dehydrase was photoirradiated under identical conditions and found to sustain no loss of enzyme activity or change in its UV spectrum (data not shown).

The structure of the inactivator moiety of DUO-inactivated dehydrase both before and after photoirradiation was probed by ^{13}C NMR spectroscopy, and the results were interpreted by comparisons to NMR data from model compounds (see Discussion). Samples of [1-^{13}C]-, [2-^{13}C]-, and [3-^{13}C]DUO were synthesized and incubated with dehydrase. The ^{13}C NMR spectra of [1-^{13}C]DUO, native dehydrase, [1-^{13}C]DUO-inactivated

(6) Endo, K.; Helmkamp, G. M., Jr.; Bloch, K. *J. Biol. Chem.* **1970**, *245*, 4293–4296.
(7) Cronan, J. E., Jr.; Li, W.-B.; Coleman, R.; Narasimhan, M.; de Mendoza, D.; Schwab, J. M. *J. Biol. Chem.* **1988**, *263*, 4641–4646.
(8) Bevitt, D. J.; Cortes, J.; Haydock, S. F.; Leadlay, P. F. *Eur. J. Biochem.* **1992**, *204*, 39–49.
(9) Henderson, B. S.; Haridas, K.; Schwab, J. M. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1381–1384.
(10) Schwab, J. M.; Li, W.-b.; Ho, C.-K.; Townsend, C. A.; Salituro, G. M. *J. Am. Chem. Soc.* **1984**, *106*, 7293–7294.
(11) Bassani, R.; DiFuria, F.; Curci, R. *Spectrosc. Lett.* **1974**, *7*, 531–536.
(12) Hanna, M. M. *Methods Enzymol.* **1989**, *180*, 383–409.
(13) Bayley, H.; Staros, J. V. In *Azides and Nitrenes: Reactivity and Utility*; Scriven, E. F. V., Ed.; Academic Press, Inc.: Orlando, FL, 1984; pp 433–490.

Scheme 3

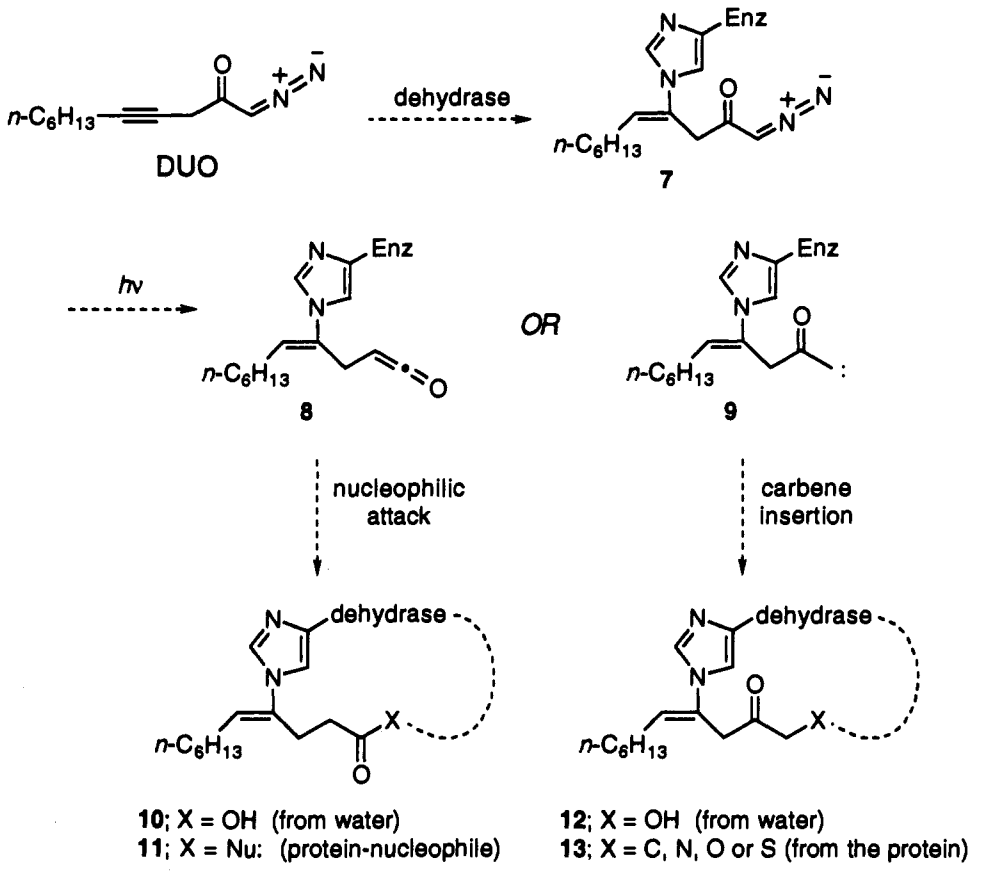


Table 1. Amino Acid Analyses

dehydrase sample	histidine residues/subunit
native	1.96 ± 0.03[a]
DUO-inactivated	1.63 ± 0.02[a]
DUO-inactivated, photoirradiated	1.23 ± 0.01[b]
3-decynoyl-inactivated	1.17 ± 0.03[b]

[a] Based on four samples. [b] Based on two samples.

dehydrase, and photoirradiated, [1-^{13}C]DUO-inactivated dehydrase are shown in Figure 3. Carbon-1 of [1-^{13}C]DUO resonates at 57.1 ppm, a value that is well within the range for α-carbons of other α-diazocarbonyl compounds.[14] Since following enzyme inactivation and removal of excess inactivator by dialysis this signal is shifted only slightly, to 60.7 ppm, it can be concluded that the α-diazoketone function remains intact. After photoirradiation, the signal at 60.7 disappears, and a new, broad peak appears at 36 ppm.

The ^{13}C NMR spectra of [2-^{13}C]DUO, native dehydrase, [2-^{13}C]DUO-inactivated dehydrase, and photoirradiated, [2-^{13}C]-DUO-inactivated dehydrase are shown in Figure 4. Carbon-2 of [2-^{13}C]DUO resonates at 193.8 ppm. Following enzyme inactivation and removal of excess inactivator by dialysis, a new, slightly shifted signal is observed at 191.5 ppm. Again, this small perturbation is consistent with retention of the α-diazoketone function. After photoirradiation, the signal at 191.5 disappears and is replaced by a peak at 179.2 ppm. The latter chemical shift is typical of the carbonyl of a carboxylic acid or a derivative thereof.[15]

The ^{13}C NMR spectra of [3-^{13}C]DUO, native dehydrase, [3-^{13}C]DUO-inactivated dehydrase, and photoirradiated, [3-^{13}C]-DUO-inactivated dehydrase are shown in Figure 5. Carbon-3 of [3-^{13}C]DUO resonates at 30.2 ppm. Following enzyme inactivation and removal of excess inactivator by dialysis, a new signal is observed at 41.8 ppm. After photoirradiation, this signal disappears, and a new, broad peak appears at 26 ppm. The

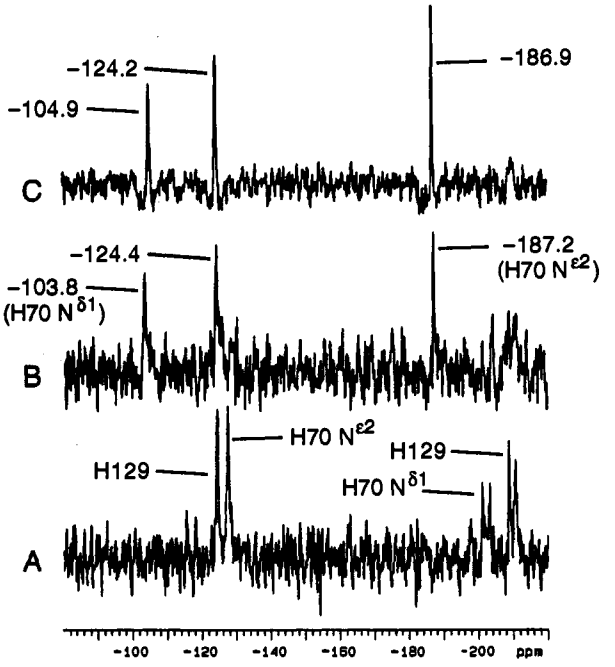


Figure 1. Imidazole nitrogen region of ^{15}N NMR spectra (50 MHz) of (A) native [U-^{15}N]dehydrase; (B) [U-^{15}N]dehydrase inactivated with 3-decynoyl-NAC; and (C) [U-^{15}N]dehydrase inactivated with DUO. The signals at −124.4 and −210 ppm have been assigned previously to H129; the other two signals are from H70.[5] That the two high-field signals in spectrum A appear as doublets results from incomplete proton decoupling.[5] Chemical shifts are referenced to external $H^{15}NO_3$ (1.0 M), which was set to 0.0 ppm. Spectra A and B are adapted from ref 5.

(14) Regitz, M.; Maas, G. *Diazo Compounds: Properties and Synthesis*; Academic Press, Inc.: Orlando, FL, 1986; pp 48, 49.

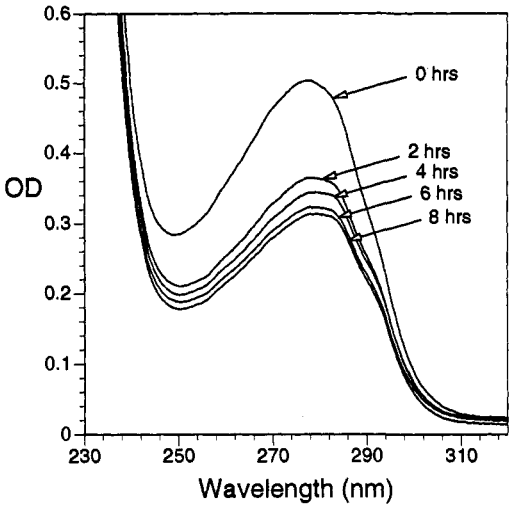


Figure 2. Time course for photoirradiation of DUO-inactivated dehydrase.

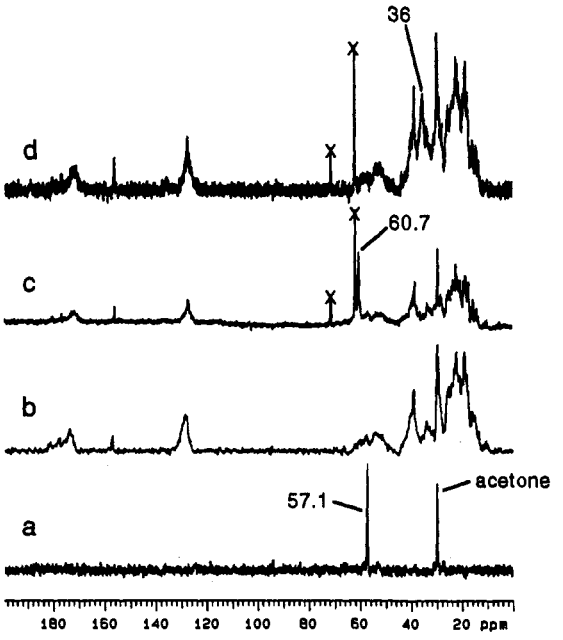


Figure 3. Proton-decoupled ^{13}C NMR spectra depicting the modification of dehydrase by [1-^{13}C]DUO: (a) [1-^{13}C]DUO; (b) native dehydrase (40 mg/mL; 24 832 transients); (c) [1-^{13}C]DUO-inactivated dehydase (24 mg/mL; 58 880 transients); and (d) photoirradiated, [1-^{13}C]DUO-inactivated dehydrase (14 mg/mL; 58 880 transients). The sharp signals at 62.1 and 71.8 ppm marked by the symbol "×" in spectra c and d are due to glycerol contamination resulting from the method of sample preparation. A small amount of acetone (ca. 1% v/v; δ 29.8 ppm) is present in all samples.

interpretation of these chemical shift values is presented in the Discussion section.

While the data obtained from the ^{13}C NMR experiments were interpreted in terms of a photochemical Wolff rearrangement and attack of a nucleophile on the resulting ketene (**8**; see Discussion), the identity of the nucleophile was unknown. Peptide mapping and sequencing experiments were conducted in order to differentiate between (a) protein cross-linking via intramolecular nucleophilic attack on the ketene and (b) nucleophilic attack by water (cf. **10** or **11**).

Dehydrase was inactivated with [6-^{3}H]DUO (synthesized as shown in Scheme 4), and the inactivated enzyme was photoirradiated as before. The radiolabeled protein was divided into

(15) Levy, G. C.; Lichter, R. L.; Nelson, G. L. *Carbon-13 Nuclear Magnetic Resonance Spectroscopy*, 2nd ed.; John Wiley and Sons: New York, 1980; Chapter 3.

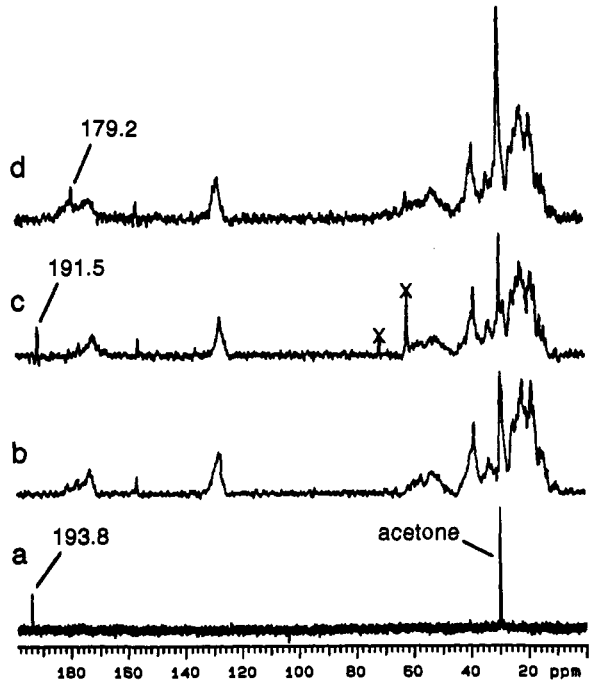


Figure 4. Proton-decoupled ^{13}C NMR spectra depicting the modification of dehydrase by [2-^{13}C]DUO: (a) [2-^{13}C]DUO; (b) native dehydrase (40 mg/mL; 24 832 transients); (c) [2-^{13}C]DUO-inactivated dehydase (25 mg/mL; 15 360 transients); and (d) photoirradiated, [2-^{13}C]DUO-inactivated dehydrase (20 mg/mL; 37 376 transients; 35 000-Hz spectral window). The sharp signals at 62.1 and 71.8 ppm marked by the symbol "×" in spectrum c are due to glycerol contamination resulting from the method of sample preparation. A small amount of acetone (ca. 1% v/v; δ 29.8 ppm) is present in all samples.

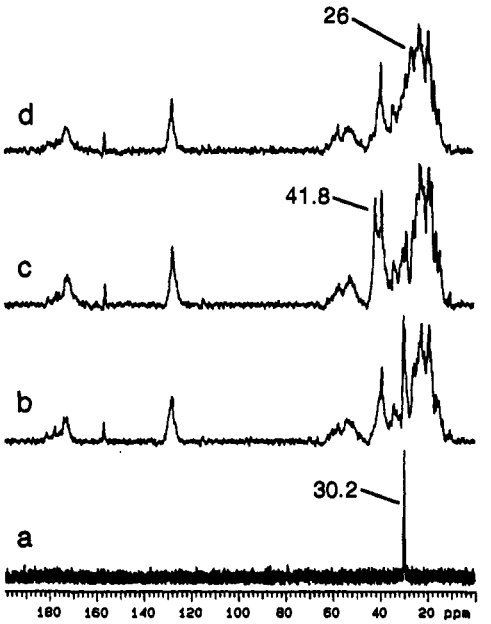


Figure 5. Proton-decoupled ^{13}C NMR spectra depicting the modification of dehydrase by [3-^{13}C]DUO: (a) [3-^{13}C]DUO; (b) native dehydrase (40 mg/mL; 24 832 transients); (c) [3-^{13}C]DUO-inactivated dehydrase (38 mg/mL; 41 984 transients); and (d) photoirradiated, [3-^{13}C]DUO-inactivated dehydrase (29 mg/mL; 37 376 transients). A small amount of acetone (ca. 1% v/v; δ 29.8 ppm) is present in the native dehydrase sample but not in the other samples.

portions that were incubated with either trypsin or chymotrypsin, and the proteolytic digests were fractionated by reversed-phase HPLC. The results are summarized in Table 2. In subsequent HPLC runs, the radiolabeled fragments were collected and subjected to automated Edman sequencing. In each peptide,

Scheme 4

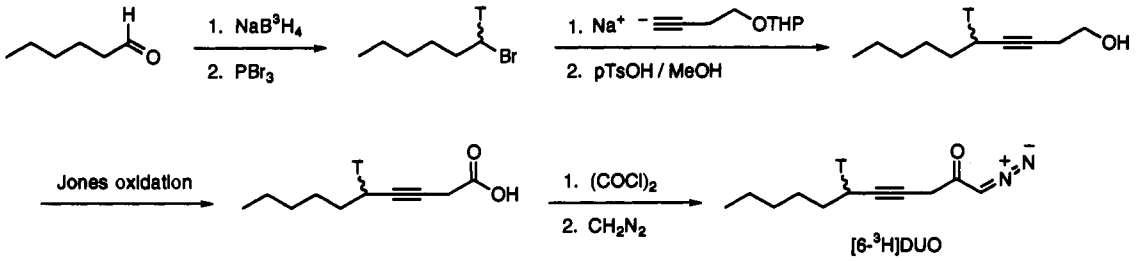


Table 2. Separation and Characterization of Tritiated Proteolytic Fragments

peptide number	protease	retention time (min)[a]	dpm[b]	partial sequence[c]	mass[d]	identity of peptide
c1	chymotrypsin	29.2	2550	GCXF	703.2	C_{68}-F_{71}
c2	chymotrypsin	32.2	1257	GXXF	645.2	G_{68}–F_{71}[e]
c3	chymotrypsin	42.3	1092	FGCXF	850.0	F_{67}-F_{71}
c4	chymotrypsin	57.3	5452	FFGCXF	997.0	F_{66}–F_{71}
t1	trypsin	26.3	1249	FFGCXFI ...	2039.6	F_{66}–K_{81}
t2	trypsin	30.2	536	GCXFIxxxVMPG ...	2423.9	G_{68}–G_{79}
t3	trypsin	43.2	3499	FFGCXFI	2714.8	F_{66}–I_{72}

[a] Derivatized peptides were purified by reverse-phase HPLC, as described in the Experimental Section. [b] One-milliliter fractions of the effluent were analyzed by liquid scintillation counting. For broad peaks, this value is estimated from the sums of the dpm values of adjacent fractions. A background value of 130 dpm (chymotryptic peptides) or 200 (tryptic peptides) has been substracted from the number of dpm observed for each fraction. [c] From Edman sequencing. Cysteine was identified as carboxymethylcysteine. The symbol "X" indicates that no residue was detected in this sequencing cycle. The symbol "x" indicates a sequencer malfunction during this cycle. [d] Peptides c1–c4 were analyzed by fast atom bombardment mass spectrometry (FAB-MS), and peptides t1–t3 were analyzed by plasma desorption mass spectrometry (PDMS). The values reported are for the protonated molecular ions (MH^+). [e] Identical with peptide c1, except for cysteine rather than carboxymethylcysteine.

Edman analysis failed to detect an amino acid at position 70, which is histidine in the native enzyme. No other sequence abnormalities were observed.

The radiolabeled fragments were also analyzed by mass spectrometry (Table 2), which enabled the identities of peptides c1–c4 and t3 to be confirmed, provided complete sequences for peptides t1 and t2, and gave an indication of the fate of the photoirradiated diazoketone moiety. The experimentally determined masses reported in Table 2 are equal in each case to the mass of the peptide plus the mass of the inactivator moiety. For the various peptides, the difference between the observed mass and that which is calculated for the peptide portion alone was 181.6–189. The predicted molecular mass for the inactivator moiety of **10**, in which ketene **8** has suffered nucleophilic attack by water, is 182, while that of **11**, which would result from attack by a protein nucleophile, is 164. The experimental results clearly are most consistent with adduct structure **10**.

The possibility was also considered that a cross-linked adduct (**11**) might be formed from ketene **8** initially, but that the "X to carbonyl" bond is hydrolyzed, either by the protease or chemically, during purification of fragment peptides by reversed-phase HPLC. In an effort to test this hypothesis, samples of dehydrase were analyzed by electrospray ionization mass spectrometry (ESMS).[16] Accurate molecular weights for intact protein molecules can be obtained by ESMS, with minimal sample preparation. The major component in the mass spectrum of native, wild-type dehydrase was found to have a molecular mass of 18 837 ± 2, which is in excellent agreement with the calculated value of 18 838.[5,7,17]

The mass of the major component in the ES mass spectrum of DUO-inactivated dehydrase was found to be 19 031 ± 3, which is in excellent agreement with the calculated value of 19 030. The observed mass of photoirradiated, DUO-inactivated dehydrase is 19 024 ± 4. The value expected for **10** is 19 020, while that expected for a cross-linked adduct (**11**) is 19 002. Clearly, the experimental result is most consistent with structure **10**.

Discussion

Although it is doubtful that H70 alone is responsible for all of the acid–base chemistry associated with dehydrase-catalyzed equilibration of **1–3** (see below), efforts thus far to identify amino acids other than H70 at dehydrase's active site have been unsuccessful. DUO was designed to address this need, since its photoreactive diazoketone moiety should be positioned near the substrate carbonyl binding site rather than in the vicinity of H70. Furthermore, since DUO is tethered to H70 at the time of photoirradiation, low selectivity should not be a problem as it is with many other photoaffinity reagents.[13,18]

Kinetic analyses have shown that DUO is, as expected originally, a mechanism-based inactivator of dehydrase.[9] The ^{15}N and ^{13}C NMR data that are presented in this paper now show that in the nonphotochemical inactivation event H70 is alkylated by DUO and that the diazoketone moiety is unaffected (cf. **7**). This conclusion is based upon (a) the obvious similarity between the imidazole regions of the ^{15}N NMR spectra of 3-decynoyl-NAC- and DUO-inactivated dehydrase (Figure 1) and (b) the minor chemical shift changes experienced by carbons 1 and 2 upon inactivation of dehydrase by DUO but prior to photoirradiation (spectra a and c in Figures 3 and 4). It is also supported by the fact that photoirradiation leads to substantial changes in the UV and NMR spectra of the enzyme–inactivator adduct (Figures 2–5).

The ^{13}C NMR data are most consistent with the hypothesis that upon photoirradiation the diazoketone moiety of DUO-inactivated dehydrase undergoes a Wolff rearrangement to a ketene (**8**), which is attacked by a nucleophile to give **10** or **11**. This conclusion is based largely upon comparisons between the chemical shifts of the ^{13}C-enriched carbons (see spectrum d in

(16) Mann, M. *Org. Mass Spectrom.* **1990**, *25*, 575–587.

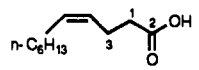
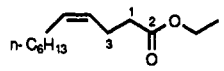
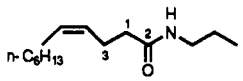
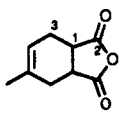
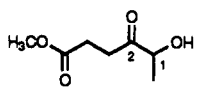
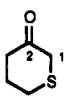
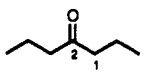
(17) In addition to the major mass peak at 18 837, a minor component (ca. 30%) was observed, at mass 18 969. The same ratio of protein components was observed in the mass spectra of the derivatized dehydrase samples. It was subsequently found by Edman analysis (data not shown) that wild-type dehydrase overexpressed in *E. coli* strain RA224[5] is contaminated to the extent of ca. 30% with protein retaining the *N*-terminal methionine residue. This would increase the molecular mass by 131.

(18) Bayley, H.; Knowles, J. R. *Methods Enzymol.* **1977**, *46*, 69–114.

(19) *Sadtler Standard Carbon-13 NMR Spectra*; Sadtler Research Laboratories, Inc.: Philadelphia, 1976.

(20) Johnson, L. F.; Jankowski, W. C. *Carbon-13 NMR Spectra: A Collection of Assigned, Indexed, and Coded Spectra*; John Wiley & Sons: New York, 1972.

Table 3. Model Compounds for the Photoirradiated DUO–Dehydrase Adduct: ^{13}C NMR Data

compound[a]	compd number	reactive intermediate modeled	"X" group modeled	chemical shift (ppm) C-1	C-2	C-3
photoirradiated DUO–dehydrase adduct	**10–13**?	N/A	N/A	36	179.2	26
	**14**	ketene	H_2O	34.2	179.9	22.6
	**15**	ketene	serine threonine (tyrosine)	34.4	173.1	22.8
	**16**	ketene	lysine	36.6	172.4	23.5
	**17**[b]	ketene	aspartic acid glutamic acid	39.6	174.8	26
	**18**[b]	α-ketocarbene	H_2O	72.9	211.4	*d*
	**19**[b]	α-ketocarbene	lysine	66.4	205.9	*d*
	**20**[b]	α-ketocarbene	cysteine	41.8	203.6	*d*
	**21**[c]	α-ketocarbene	(C–H bond insertion)	44.7	210.6	*d*

[a] For ease of comparison with the data for the photoirradiated DUO–dehydrase adduct, an unconventional numbering scheme, based upon the carbon atom of DUO from which each carbon of the adduct would have arisen, is used for compounds **14–17**. [b] ^{13}C NMR data taken from ref 19. [c] ^{13}C NMR data taken from ref 20. [d] The chemical shift value for this carbon atom is not likely to be useful for comparison with the photoirradiated DUO–dehydrase adduct.

Figures 3–5 and Table 3) and those of the appropriate carbon atoms of model compounds (Table 3). To understand the analysis, it is important to note that due to the Wolff rearrangement, carbons 1, 2, and 3 of **10** or **11** would come from carbons 2, 1, and 3, respectively, of DUO. The carbon skeletons of **12** and **13** would not be rearranged, however. Compounds **14–17** are models for rearranged enzyme–DUO adducts **10** and **11**, while **18–21** are models for **12** and **13**.

The complexity of the upfield regions of the spectra in Figures 3 and 5 is such that unequivocal structure assignments cannot be made in the absence of other data. Still, meaningful conclusions may be drawn from the chemical shifts of the carbonyls (Figure 4), which appear in the relatively uncluttered downfield region. It is immediately evident that the carbonyl carbons ("C-2" in Table 3) of ketones **18–21** resonate at much lower field (ca. 25–30 ppm) than does the carbonyl of the photoirradiated DUO–dehydrase adduct. On the other hand, all of the NMR data for **14–17** are close to those for the photoirradiated DUO–dehydrase adduct. Thus, the carbene insertion hypothesis (**7** → **9** → **12** or **13**) can be ruled out, and the adduct can now be assigned structure **10** or **11**, which results from Wolff rearrangement of **7**, followed by nucleophilic attack on ketene **8**. Interestingly, the chemical shift of 26 ppm for C-3 of adduct **10**/**11** indicates that C-3 is allylic (rather than vinylic), which means that adduct **7** does not isomerize to its 2,3-double-bond isomer. This behavior contrasts with the spontaneous isomerization of 3-decynoyl-NAC–dehydrase adduct **6** to its conjugated, 2,3-double-bond isomer.[4,10] The failure of **7** to isomerize may reflect the rather high electron density at the carbonyl carbon of an α-diazoketone, in which negative charge is delocalized from the α-carbon into the carbonyl group.[14] Thus, enolization is discouraged.

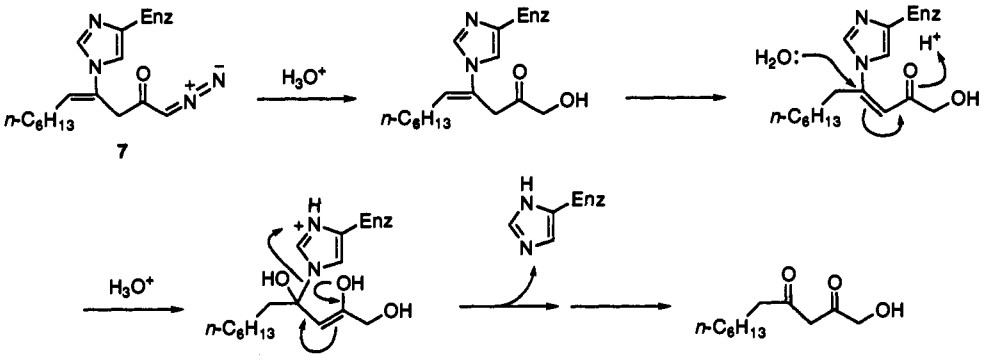
Initially, the amino acid analysis results (Table 1) were difficult to interpret. However, the ^{15}N NMR spectra in Figure 1 provide solid evidence for the stoichiometric formation of an adduct between DUO and H70. The unexpectedly high value for unmodified histidine in the hydrolysate from DUO-inactivated dehydrase now can be rationalized by the release of H70 under the conditions used for acid hydrolysis, as illustrated in Scheme 5.[21] Note that the first step proposed is the acid-mediated conversion of the α-diazoketone to the α-hydroxyketone, which removes the electronic barrier to carbon–carbon double-bond rearrangement. A Michael addition–elimination sequence on the conjugated enone leads to the release of unmodified histidine.

If the release of free histidine by acid hydrolysis of DUO-inactivated dehydrase does follow the mechanism shown in Scheme 5, then why doesn't the same thing happen with 3-decynoyl-NAC–inactivated dehydrase? Indeed, it has long been recognized that vinylimidazoles, including the 3-decynoyl-NAC–dehydrase adduct (**6**), tend to be unusually stable toward hydrolysis in either strong or acid or strong base.[22,23] Hupe et al. had regarded vinylimidazoles as enamines and interpreted their hydrolytic stability in terms of the failure of the imidazole moiety to participate in β-carbon protonation (e.g., carbon-4 of adduct **6**).[23] The iminium ion intermediate (which would be a diazafulvenium species) is not formed since the heterocycle would be antiaromatic. Still, one might expect adduct **6** to hydrolyze in strong acid by the mechanism shown in Scheme 5. A hypothesis for its failure

(21) The mechanism portrayed in Scheme 5 is speculative, and a number of variations can be envisioned.

(22) Morisaki, M.; Bloch, K. *Biochemistry* **1972**, *11*, 309–314.
(23) Hupe, D. J.; Kendall, M. C. R.; Spencer, T. A. *J. Am. Chem. Soc.* **1972**, *94*, 1254–1263.

Scheme 5



to do so is that the thiol ester moiety of **6** (or of the isomeric, conjugated enoyl thiol ester) hydrolyzes rapidly and that the resulting carboxylic acid is less susceptible to Michael attack by water than is an enone.

While DUO–dehydrase adduct **7** hydrolyzes in strong acid, the *photoirradiated* form of this adduct (**10**) is hydrolytically stable. This observation is understood readily, since **10** itself is not a Michael acceptor, and even if the carbon–carbon double bond were to rearrange into conjugation with the carbonyl, the imidazole ring would be out of position for an addition–elimination mechanism.

The hydrolytic lability of DUO-inactivated dehydrase demonstrates that given a proper array of functional groups, there can be more than one mechanism for hydrolysis of a vinylimidazole.

The similarities of all of the chemical shift data in Table 3 for compounds **14–17** and for the photoirradiated DUO–dehydrase adduct preclude any insight as to the nature of the attacking nucleophile. For this purpose, it was necessary to turn to peptide mapping, peptide sequencing, and mass spectrometry, which provide firm proof that ketene **8** undergoes nucleophilic attack by water, rather than by a protein nucleophile. The failure to detect, by sequencing, any cross-linked peptides in the proteolytic digests of photoirradiated, DUO-inactivated dehydrase is consistent with this conclusion, and the sequencing results are supported by the mass spectrometric analyses of the peptides (Table 2). The most convincing evidence, however, comes from the analysis of intact protein samples by electrospray ionization mass spectrometry (ESMS). The beauty of ESMS for the present application is that sample preparation is exceedingly gentle, requiring only the desalting of the protein sample by reversed-phase HPLC. Furthermore, the range of masses that can be analyzed by ESMS extends well past that of dehydrase, and the resolution is such that the mass difference of 18 between **10** and **11** can be distinguished.

Clearly, DUO's alkylation chemistry and photochemistry proceed according to plan. However, while DUO was envisioned as a protein cross-linking agent, the only amino acid that is modified by DUO is H70, a residue that long has been known to be at dehydrase's active site. Initially, this outcome was disappointing, since it seemed to provide no further information about dehydrase's active site. Also, the fact that the photochemically generated ketene, **8**, reacts with water appeared to be the trivial result, since dehydrase is surrounded by bulk water, and the propensity of reactive intermediates generated from photoaffinity reagents to react with bulk water is well-known.[13,18,24,25] The foregoing notwithstanding, recent discussions with our collaborators (Janet Smith and Minsun Leesong, Department of Biological Sciences, Purdue University) who are pursuing the X-ray crystal structure of 3-decynoyl-NAC–inactivated dehydrase have cast our results with DUO in a much different light. First, their preliminary interpretation of the electron density map indicates that the two identical active sites per protein dimer are extremely well shielded from bulk water. Second, there is space for a number of ordered water molecules within the protein, notably in the immediate vicinity of each active site. While the presence of ordered water molecules at the active sites is still unconfirmed and their specific functions are unknown, reasonable suggestions can be made. For instance, the water molecule that is eliminated from substrate **1** may be bound at the active site. Also, one or more water molecules may bridge (by hydrogen bonding) between the substrate (or inactivator) and a specific amino acid residue and be involved in substrate binding or catalysis. In fact, the preliminary interpretation of the electron density map suggests that there is a water molecule in the general vicinity of both the inactivator's sulfur atom and the hydroxyl group of Y155. It is conceivable that this water molecule (rather than a tyrosine residue alone) serves as an electrophilic catalyst of substrate deprotonation (see below). In this light, it is realistic to suggest that ordered water at the active site may be close enough to C-1 of ketene **8** to react with it.

There has been considerable discussion in the literature recently concerning mechanisms of enzyme-catalyzed carbon acid deprotonation, since deprotonation of a carbonyl compound to give the enolate is energetically demanding and therefore slow. Addressing this issue, Gerlt and his collaborators have argued convincingly that concerted enolization, catalyzed by a general acid (referred to by these authors as an "electrophilic catalyst") as well as a general base, is favorable energetically and for this reason is common in nature.[26–29] Apropos of the suggestion that ordered water is present at dehydrase's active site, Liu and Santi have postulated that an ordered water molecule at the active site of thymidylate synthase acts as an electrophilic catalyst for substrate enolization.[30] Very recently, Guthrie and Kluger have proposed electrostatic stabilization of enolate anions as an alternative mechanism for promoting the enzyme-catalyzed deprotonation of carbon acids.[31]

For the following reasons electrophilic catalysis or electrostatic stabilization must be invoked to explain substrate deprotonation by the *E. coli* dehydrase. All of the available evidence points toward H70 as the sole active site base that mediates deprotonation of the thiol ester substrates.[2–5] Of dehydrase's three substrates, **3**, which is arguably the most acidic, is still a weak acid, with a

(24) Shafer, J.; Baronowsky, P.; Laursen, R.; Finn, F.; Westheimer, F. H. *J. Biol. Chem.* **1966**, *241*, 421–427.
(25) Chowdhry, V.; Vaughan, R.; Westheimer, F. H. *Proc. Natl. Acad. Sci. U.S.A* **1976**, *73*, 1406–1408.
(26) Gerlt, J. A.; Gassman, P. G. *J. Am. Chem. Soc.* **1993**, *115*, 11552–11568.
(27) Gerlt, J. A.; Gassman, P. G. *Biochemistry* **1993**, *32*, 11943–11952.
(28) Gerlt, J. A.; Kozarich, J. W.; Kenyon, G. L.; Gassman, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 9667–9669.
(29) Gerlt, J. A.; Gassman, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 5928–5934.
(30) Liu, L.; Santi, D. V. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8604–8608. We thank one of the referees for bringing this paper to our attention.
(31) Guthrie, J. P.; Kluger, R. *J. Am. Chem. Soc.* **1993**, *115*, 11569–11572.

pK_a estimated at 13–15.[32] Substrate **1** should be considerably less acidic than **3**; its C-2 protons may be comparably acidic to those of cyclohexanone (pK_a = 18.1[36]). ^{15}N NMR experiments have suggested that H70 of dehydrase is an especially weak base, with an imidazolium pK_a < 5;[5] thus, it is unlikely that H70 alone could deprotonate **1**–**3** at a substantial rate. A major goal of ongoing structure and function studies of dehydrase will continue to be the identification of features that facilitate substrate deprotonation.

Experimental Section

All reagents were of reagent grade unless otherwise indicated. Benzene was distilled from sodium and benzophenone under nitrogen. Diethyl ether was distilled from lithium aluminum hydride under nitrogen. NMR spectra were obtained on a Chemagnetics A200 or a Varian VXR-500 spectrometer, and $CDCl_3$ was used as the solvent for all samples unless otherwise indicated. Unless otherwise noted, ^{1}H NMR spectra are referenced either to tetramethylsilane (0.0 ppm) or to the residual $CHCl_3$ in the solvent (7.24 ppm), and ^{13}C NMR spectra are referenced to $CDCl_3$ (77.0 ppm). Infrared spectra were obtained on a Perkin-Elmer Model 1600 FT-IR, and samples were prepared as solutions in high-purity CCl_4. Flash chromatography was performed on silica gel 60, 230–400 mesh (EM Science), and thin-layer chromatography (TLC) utilized silica gel GHLF plates (Analtech). TLC plates routinely were visualized with ultraviolet light or with ethanolic phosphomolybdic acid. TLC radiochromatograms were analyzed using a Berthold Tracemaster 20 Scanner with 10% methane/90% argon as the counting gas. Gas chromatography was performed on a Hewlett-Packard 5890 equipped with a 15 m × 530 μm DB-WAX column (J&W Scientific). HPLC was carried out on a system that included a Beckman 168 diode array detector and two Beckman 110A pumps, controlled via Beckman System Gold V. 5.1 software. Liquid scintillation counting utilized a Beckman LS 1801 scintillation counter. Fast atom bombardment mass spectrometry (FAB-MS) was conducted on a Kratos MS-50 high-resolution mass spectrometer. Plasma desorption mass spectra were obtained on a BioIon 20R mass spectrometer (BioIon KB, Uppsala, Sweden).

1-Diazo-4-undecyn-2-one (DUO). The procedure used for the synthesis of diazoketones is essentially the same as that reported by Rosenquist and Chapman.[37] 3-Decynoic acid (1.00 g; 5.95 mmol), prepared by Jones oxidation[38,39] of 3-decyn-1-ol,[4] was treated with oxalyl chloride (0.77 g; 6.0 mmol) in CH_2Cl_2 (10 mL) for 16 h at room temperature under nitrogen. Ethereal CH_2N_2[40] (ca. 5 equiv) was added to the crude acid chloride at 0 °C, and the reaction mixture was stirred at 0 °C for 15 min. Excess CH_2N_2 and the solvent were removed by application of a gentle stream of nitrogen. The diazoketone was purified by flash chromatography (1:10 EtOAc/petroleum ether), yielding 756 mg (66%) of a bright yellow, viscous liquid. ^{1}H NMR (500 MHz): δ 0.87 (t, J = 7.1 Hz, 3 H, CH_3), 1.2–1.4 (m, 6 H, CH_2), 1.49 (m, 2 H, $CH_2CH_2C{\equiv}C$), 2.18 (tt, J = 7.2 Hz, J = 2.5 Hz, 2 H, $CH_2CH_2C{\equiv}C$), 3.16 (t, J = 2.5 Hz, 2 H, CH_2CO), 5.81 (s, 1 H, CHN_2). ^{13}C{^{1}H} NMR (125 MHz): δ 189.0, 85.0, 73.2, 54.1, 31.4, 29.0, 22.7, 18.8, 14.1. IR: 2960, 2922, 2853, 2108, 1736, 1652, 1451, 1410, 1374, 1348, 1251, 1102, 1012 cm^{-1}. UV (3:1 CH_3CN/H_2O): λ_{max} 250, 272 nm.

1-Diazo-4-[1-^{13}C]undecyn-2-one ([1-^{13}C]DUO). Decynoic acid (67 mg; 0.40 mmol) and 60 μL of ($COCl_2$) were stirred in 5 mL of anhydrous Et_2O for 16 h at 25 °C under an atmosphere of N_2. The resulting acid chloride, which had been concentrated *in vacuo*, was added to an ethereal solution, cooled to 0 °C, of $^{13}CH_2N_2$, prepared from 117 mg (0.50 mmol) of *N*-[^{13}C]methyl-*N*-nitroso-*p*-toluenesulfonamide (99 atom % ^{13}C).[40] After 15 min the residual $^{13}CH_2N_2$ was removed by the gentle application of a stream of N_2, and the crude product was concentrated under reduced pressure. Flash chromatography (1:9 EtOAc/hexanes) yielded 31 mg (40%) of the desired product. ^{1}H NMR (500 MHz): δ 0.87 (t, J = 7.1 Hz, 3 H, CH_3), 1.2–1.4 (m, 6 H, CH_2), 1.49 (m, 2 H, $CH_2CH_2C{\equiv}C$), 2.18 (tt, J = 7.2 Hz, J = 2.5 Hz, 2 H, $CH_2CH_2C{\equiv}C$), 3.16 (t, J = 2.5 Hz, 2 H, CH_2CO), 5.81 (d, J_{CH} = 101.7 Hz, 1 H, CHN_2). ^{13}C{^{1}H} NMR (125 MHz): δ 54.0 ($CDCl_3$) (δ 57.1 in 10 mM KPO_4, 1 μM EDTA, 10% D_2O, pH 7.0).

3-[1-^{13}C]Decynoic Acid. The title compound was prepared by a route analogous to that employed for the preparation of 3-[2-^{13}C]decynoic acid.[4] Starting from 640 mg (3.15 mmol) of 1-bromo-2-nonyne and 610 mg (6.74 mmol) of Cu^{13}CN (prepared[41] from Na^{13}CN; 98.4 atom % ^{13}C), the purified, labeled carboxylic acid (94 mg) was obtained in 17.8% overall yield. ^{1}H NMR (500 MHz): δ 0.87 (t, J = 6.9 Hz, 3 H, CH_3), 1.22–1.39 (m, 6 H, CH_2), 1.49 (m, 2 H, $CH_2CH_2C{\equiv}C$), 2.18 (tt, J = 7.2 Hz, J = 2.5 Hz, 2 H, $CH_2CH_2C{\equiv}C$), 3.31 (t, J = 2.4 Hz, 2 H, CH_2CO). ^{13}C{^{1}H} NMR (125 MHz): δ 173.6.

1-Diazo-4-[2-^{13}C]undecyn-2-one ([2-^{13}C]DUO). The title compound was prepared from 3-[1-^{13}C]decynoic acid by the procedure used to prepare unlabeled DUO. From 94 mg (0.56 mmol) of 3-[2-^{13}C]decynoic acid was obtained 52 mg (48%) of the labeled diazoketone. ^{1}H NMR (500 MHz): δ 0.87 (t, J = 7.0 Hz, 3 H, CH_3), 1.20–1.41 (m, 6 H, CH_2), 1.49 (m, 2 H, $CH_2CH_2C{\equiv}C$), 2.17 (m, 2 H, $CH_2CH_2C{\equiv}C$), 3.16 (d, J = 3.5 Hz, 2 H, CH_2CO), 5.81 (d, J = 2.1 Hz, 1 H, CHN_2). ^{13}C{^{1}H} NMR (125 MHz): δ 190.0 ($CDCl_3$) (δ 193.8 in 10 mM KPO_4, 1 μM EDTA, 10% D_2O, pH 7.0).

1-Diazo-4-[3-^{13}C]undecyn-2-one ([3-^{13}C]DUO). The title compound was prepared from 3-[2-^{13}C]decynoic acid[4] by the procedure used to prepare unlabeled DUO. From 10 mg of 3-[2-^{13}C]decynoic acid (99.0 atom % ^{13}C) was obtained 7.6 mg (66%) of the labeled diazoketone. ^{1}H NMR (500 MHz): δ 0.87 (t, J = 7.1 Hz, 3 H, CH_3), 1.2–1.4 (m, 6 H, CH_2), 1.49 (m, 2 H, $CH_2CH_2C{\equiv}C$), 2.18 (m, J = 7.2 Hz, 2 H, $CH_2CH_2C{\equiv}C$), 3.16 (d, J_{CH} = 132 Hz, 2 H, CH_2CO), 5.81 (s, 1 H, CHN_2). ^{13}C{^{1}H} NMR (125 MHz): δ 32.0 ($CDCl_3$) (δ 30.0 in 10 mM KPO_4, 1 μM EDTA, 10% D_2O, pH 7.0).

(*Z*)-1-Diazo-4-undecen-2-one (11). (*Z*)-3-Decenoic acid (100 mg; 0.59 mmol), prepared by Jones oxidation[38,39] of 3-decen-1-ol,[4] was stirred for 16 h at 23 °C with oxalyl chloride (100 μL; 1 mmol) in CH_2Cl_2 (5 mL). Following removal of the solvent *in vacuo*, the crude acid chloride was reacted with ethereal CH_2N_2 (3 equiv) at −73 °C for 0.5 h. After concentration of the reaction mixture, flash chromatography (1:9 petroleum ether/EtOAc) of the residue yielded 80 mg (70%) of the diazoketone as a yellow oil. ^{1}H NMR (500 MHz): δ 0.83 (t, J = 6.9 Hz, 3 H, CH_3), 1.17–1.35 (m, 8 H, CH_2), 1.99 (dt, J = 7.2 Hz, J = 7.2 Hz, 2 H, $CH_2CH_2CH{=}CH$), 3.03 (d, J = 6.0 Hz, 2 H, $CHCH_2CO$), 5.28 (s(br), 1 H, CHN_2), 5.45 (m, 1 H, $CH_2CH_2CH{=}CH$), 5.58 (m, 1 H, $CH{=}CHCH_2CO$). ^{13}C{^{1}H} NMR (125 MHz): δ 53.9 (C-1), 39.6 (C-3), 29.1, 28.8, 27.3, 22.4, 14.0, 13.8.

(*Z*)-4-Undecenoic Acid, Ethyl Ester (15). (*Z*)-1-Diazo-4-undecen-2-one (250 mg; 0.8 mmol) was dissolved in absolute EtOH (8 mL) in a quartz photoirradiation tube. The tube was placed in a turntable reactor and photoirradiated at 5 °C using a 450-W medium-pressure Hg lamp. After 3 h, the yellow color of the starting material was no longer present, and N_2 evolution had ceased. The solvent was removed under reduced pressure, and the crude product was purified by flash chromatography (1:9 EtOAc/hexanes) to yield 125 mg (47%) of the ester as a pale yellow oil. MS (CI isobutane): m/z 213. ^{1}H NMR (500 MHz): δ 0.84 (t, J = 6.9 Hz, 3 H, $CH_3CH_2CH_2$), 1.19–1.33 (overlapping m, 11 H, CH_2, OCH_2CH_3), 2.00 (dt, J = 6.8 Hz, J = 7.1 Hz, 2 H, $(CH_2)_4CH_2CH{=}$), 2.28–2.36 (m, 4 H, CH_2CH_2CO), 4.09 (q, J = 7.2, 2 H, OCH_2), 5.25–5.32 (m, 1 H, $CH_2CH_2CH{=}$), 5.34–5.41 (m, 1 H, $=CHCH_2CO$). ^{13}C{^{1}H} NMR (125 MHz): δ 173.1, 131.4, 127.2, 60.2, 34.4, 31.8, 29.6, 29.0, 27.2, 22.8, 22.6, 14.2, 14.1.

(*Z*)-4-Undecenoic Acid (14). Compound **15** (380 mg; 1.8 mmol) was stirred for 16 h with NaOH (0.4 g; 10 mmol) in a mixture of absolute EtOH (10 mL) and water (10 mL). The reaction mixture was extracted several times with hexanes, acidified with HCl, and extracted several more times with hexanes. The second set of hexane extracts was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography (90:10:1 EtOAc/hexanes/AcOH) to yield the carboxylic acid (315 mg; 95%) as a pale yellow oil. MS (CI isobutane): m/z 185. ^{1}H NMR (500 MHz): δ 0.86

(32) Since thiol esters are considered to be about as acidic as ketones,[33] the pK_a of the C-2 protons of **3** may be close to those of 3-cyclohexenone (ca. 15.2[34]) or 5-androstene-3,17-dione (ca. 12.7[35]).

(33) Bruice, T. C.; Benkovic, S. *Bioorganic Mechanisms*; W. A. Benjamin: New York, 1966; Vol. 1, p 297.

(34) Dzingeleski, G. D.; Blotny, G.; Pollack, R. M. *J. Org. Chem.* **1990**, *55*, 1019–1023.

(35) Pollack, R. M.; Mack, J. P. G.; Eldin, S. *J. Am. Chem. Soc.* **1987**, *109*, 5048–5050.

(36) Chiang, Y.; Kresge, A. J. *Science (Washington, DC)* **1991**, *253*, 395–400.

(37) Rosenquist, N. R.; Chapman, O. L. *J. Org. Chem.* **1976**, *41*, 3326–3327.

(38) Bowers, A.; Halsall, T. G.; Jones, E. R. H.; Lemin, A. J. *J. Chem. Soc.* **1953**, 2548–2560.

(39) Heilbron, I.; Jones, E. R. H.; Sondheimer, F. *J. Chem. Soc.* **1949**, 604–607.

(40) Black, T. H. *Aldrichim. Acta* **1983**, *16*, 3–10.

(41) Reid, J. C.; Weaver, J. C. *Cancer Res.* **1951**, *11*, 188–194.

(t, J = 6.9 Hz, 3 H, CH_3), 1.20–1.35 (m, 8 H, CH_2), 2.03 (dt, J = 7.1 Hz, J = 7.1 Hz, 2 H, $(CH_2)_4CH_2CH$=), 2.28–2.42 (m, 4 H, CH_2CH_2-CO), 5.27–5.34 (m, 1 H, CH=$CHCH_2CH_2CO$), 5.38–5.45 (m, 1 H, =$CHCH_2CH_2CO$), 11.99 (br s, 1 H, OH). $^{13}C\{^1H\}$ NMR (125 MHz): δ 179.9, 131.8, 126.7, 34.2, 31.7, 29.5, 28.9, 27.2, 22.6, 14.1.

(Z)-4-Undecenoic Acid, 1-Propylamide (16). (Z)-4-Undecenoic acid (14; 98 mg; 0.53 mmol) was stirred with oxalyl chloride (75 μL; 0.87 mmol) in CH_2Cl_2 (5 mL) for 21 h. Following removal of the solvent under reduced pressure, the crude acid chloride was cooled to 0 °C and stirred with 1-aminopropane (1 mL) for 5 min. The reaction mixture was allowed to warm to room temperature, and the volatile materials were removed *in vacuo*. Flash chromatography (1:19 acetone/CH_2Cl_2) afforded the amide (120 mg; 100%) as a colorless oil. MS (CI isobutane): m/z 226. ^1H NMR (500 MHz): δ 0.82 (t, J = 6.8 Hz, 3 H, $NHCH_2CH_2CH_3$), 0.86 (t, J = 7.4 Hz, 3 H, CH_3), 1.15–1.31 (m, 8 H, CH_2), 1.46 (m, 2 H, $NHCH_2CH_2$), 1.98 (dt, J = 6.8 Hz, J_{CH} = 6.8 Hz, 2 H, $(CH_2)_4CH_2CH$=), 2.16 (t, J = 7.8 Hz, 2 H, CH_2CO), 2.31 (dt, J = 7.5 Hz, J = 7.5 Hz, 2 H, CH_2CH_2CO), 3.14 (dt, 2 H, J = 7.0 Hz, J = 7.0 Hz, $NHCH_2$), 5.23–5.30 (m, 1 H, CH=$CHCH_2CH_2CO$), 5.33–5.40 (m, 1 H, =$CHCH_2CH_2CO$), 5.81 (br s, 1 H, NH). $^{13}C\{^1H\}$ NMR (125 MHz): δ 172.4, 131.4, 127.6, 41.2, 36.6, 31.7, 29.6, 28.9, 27.2, 23.5, 22.8, 22.6, 14.0, 11.3.

1-[1-^{3}H]Hexanol. The title compound was prepared by a modification of the procedure of Parry et al.[42] used for the preparation of 5-[1-^{3}H]-hexen-1-ol. The reaction was conducted in stages, with the addition initially of a small quantity of unlabeled $NaBH_4$ to ensure that any highly reactive impurity that may be present is reduced prior to addition of NaB^3H_4. Thus, hexanal (104 mg; 1.04 mmol) was stirred with 2.98 mg (79 μmol) of unlabeled $NaBH_4$ for 3 h at room temperature in 0.1 mL of absolute EtOH. The reaction mixture was transferred (with an EtOH wash; 0.9 mL) to a vial containing NaB^3H_4 (100 mCi; 468.7 mCi/mmol; DuPont NEN Research Products), and the resulting mixture was stirred for an additional 7 h. Unlabeled $NaBH_4$ (38 mg; 0.99 mmol) was added, and the mixture was stirred at room temperature for an additional 13 h to consume the remaining aldehyde. The solvent was carefully removed *in vacuo*, and the residue was partitioned between 10% NaOH and CH_2Cl_2. The organic phase was dried over $MgSO_4$ and filtered, and the solvent was carefully removed *in vacuo* to yield 98 mg of the product as a pink oil, with a total radioactivity of 49.9 mCi (92% chemical yield; 49.9% radiochemical yield). Due to its volatility, the product was used without further purification or characterization.

1-[1-^{3}H]Bromohexane. A solution of 1-[1-^{3}H]hexanol (98 mg; 0.96 mmol; 49.9 mCi), unlabeled 1-hexanol (98 mg; 0.96 mmol), PBr_3 (344 mg; 1.27 mmol), and Et_2O (5 mL) was heated at reflux for 6 h. The reaction was terminated by the addition of ca. 5 mL of ice water. The aqueous layer was extracted with Et_2O, and the combined organic extracts were dried over $MgSO_4$. Filtration and removal of the solvent under gentle vacuum yielded the bromide (194 mg; 62%) as a clear oil. Further purification of the resulting bromide was not attempted due to its volatility.

1-[(2-Tetrahydropyranyl)oxy]-3-[5-^{3}H]decyne. The title compound was prepared by a modification of the method of Kunitani.[43] Freshly cut sodium (58 mg; 2.5 mmol) was added to a flask containing ca. 15 mL of liquid NH_3 and 2 mg of $Fe(NO_3)_3$. The solution was maintained at reflux with stirring for 1 h and then cooled to −78 °C, and 427 mg (2.77 mmol) of 1-[(2-tetrahydropyranyl)oxy]-3-butyne was added via syringe. The reaction mixture was stirred at reflux for 1.5 h and then cooled to −78 °C, and 1-[1-^{3}H]bromohexane (194 mg; 1.16 mmol) was added via syringe. The reaction mixture was stirred at reflux for an additional 3 h. Et_2O (10 mL) was added and the NH_3 allowed to evaporate. The reaction was terminated by the addition of 15 mL of saturated NH_4Cl, and the resulting mixture was extracted with Et_2O. The extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product was subjected to flash chromatography (3:97 acetone/hexanes), which yielded 20 mg of a clear oil with a total radioactivity of 0.103 mCi (chemical yield 12.5%; radiochemical yield 0.21%). This oil was contaminated with a substantial amount of unreacted 1-[(2-tetrahydropyranyl)oxy]-3-butyne. A repetition of this alkylation procedure on a similar scale (except that only 7 mL of liquid NH_3 and 135 mg of 1-[(2-tetrahydropyranyl)oxy]-3-butyne were used) gave chemical and radiochemical yields of 29 and 14%, respectively.

3-[5-^{3}H]Decyn-1-ol. A mixture of 1-[(2-tetrahydropyranyl)oxy]-3-[5-^{3}H]decyne (20 mg; 0.084 mmol) and 32 mg (0.13 mmol) of unlabeled 1-[(2-tetrahydropyranyl)oxy]-3-decyne (added to facilitate mass transfer) was stirred for 1 h in 3 mL of MeOH with 7 mg of *p*-TsOH. The reaction was stopped by the addition of 2 mL of saturated NH_4Cl, and the resulting mixture was extracted with Et_2O. The combined organic extracts were dried over $MgSO_4$ and filtered, and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (3:17 EtOAc/hexanes) to yield 25 mg (76%) of the product as a clear oil with a total radioactivity of 97 μCi (94% radiochemical yield). The major component of the product migrated as a single spot by TLC (7:33 EtOAc/hexanes) with the same R_f (0.37) as that of unlabeled material.

3-[5-^{3}H]Decynoic Acid. The title compound was made by Jones oxidation[38,39] of 3-[5-^{3}H]decyn-1-ol (25 mg, 0.15 mmol). Following four recrystallizations from hexanes, the product (12 mg; 42.5%) was obtained as white plates with a specific activity of 0.53 mCi/mmol (37% radiochemical yield).

1-Diazo-4-[6-^{3}H]undecyn-2-one ([6-^{3}H]DUO). The same methods were used as for the preparation of unlabeled DUO. From 12 mg of 3-[5-^{3}H]decynoic acid, 2.5 mg (19%) of [6-^{3}H]DUO was obtained with a total radioactivity of 7.04 μCi (19% radiochemical yield). The specific activity of [6-^{3}H]DUO was assumed to be identical to that of 3-[5-^{3}H]decynoic acid (0.53 mCi/mmol). The radiochemical purity of [5-^{3}H]DUO was >99%, as shown by (i) TLC on silica gel (1:10; EtOAc/hexanes; R_f 0.24) and (ii) HPLC (Beckman Ultrasphere ODS column, 250 × 4.6 mm; 30-min linear gradient from 1:1 water/CH_3CN to 100% CH_3CN).

General Enzymological Procedures. The *E. coli* strains DM51A[7] and RA224[5,44] were used as sources of dehydrase, which was isolated as previously.[5,7,44] [U-^{15}N]Dehydrase was obtained and purified as described by Annand.[5,44] A modification of the initial rate assay described by Kass[45] was used to determine the activity of dehydrase. Five microliters of a 20 mM stock solution of (Z)-3-decenoyl-NAC in MeOH was added to a cuvette containing 1.0 mL of 10 mM KPO_4 at 30 °C. Dehydrase was added to the cuvette, and the rate of change in absorbance at 263 nm was recorded. The initial rate was calculated as described by Kass.[45]

Protein concentrations of dehydrase solutions ordinarily were determined by the absorbance at 280 nm.[4] However, since DUO itself absorbs significantly at 280 nm, the protein content of DUO-inactivated dehydrase was determined by the Bio-Rad assay,[46] which was calibrated using a sample of native dehydrase, the concentration of which was known accurately from its absorbance at 280 nm.

Enzyme Inactivation and Photoirradiation of Inactivated Enzyme. Preparative scale inactivations were carried out such that the concentration of DUO did not exceed 300 μM, due to DUO's limited solubility in aqueous solutions. In a typical experiment, dehydrase (ca. 125 mg) was diluted to a final volume of 530 mL with 10 mM KPO_4, pH 7.0. DUO (420 μL of a 62 mg/mL solution in spectral grade acetone) was added to the enzyme solution to a final DUO concentration of 250 μM. After incubation for 8 h in the dark at room temperature, less than 1% of the starting enzyme activity remained. The solution of inactivated protein was cooled to 4 °C, concentrated to ca. 50 mL by pressure ultrafiltration in an Amicon cell, and dialyzed for 16 h against 3 × 2 L of 10 mM KPO_4, pH 7.0, to remove residual inactivator, followed by further concentration by centrifugal ultrafiltration.

Protein solutions were irradiated in a 1-L photochemical reaction vessel using a medium-pressure Hg lamp equipped with a uranium yellow filter (UV cutoff ca. 330 nm). The reaction vessel was submerged in a 4 °C water bath, and argon was bubbled gently into the bottom of the solution to effect mixing. Photoirradiation was carried out for 7–8 h, during which time the temperature of the reaction mixture increased to 16 °C. Aliquots were removed during the course of the reaction and analyzed for dehydrase activity, and ultraviolet spectra were recorded to monitor the progress of the reaction.

Protein NMR Spectroscopy. ^{15}N (50 MHz) and $^{13}C\{^1H\}$ (125 MHz) NMR spectra were obtained on protein samples in 10 mM KPO_4, 1 μM EDTA, 10% D_2O, pH 7.0, using a Varian VXR-500 spectrometer equipped with a 10-mm broad-band probe. For ^{15}N NMR, a 10-μs pulse (22° flip angle) with a 2.0-s pulse delay was used, and 16K data points were collected (zero-filled to 32K) over a spectral width of 20 000 Hz. The sample temperature was maintained at 10 °C, and gated decoupling was applied only during acquisition. The spectra were referenced to external $H^{15}NO_3$ (1.0 M, 0.00 ppm) and processed with 10-Hz line broadening. For $^{13}C\{^1H\}$ NMR, a 90° pulse (18 μs) was used with a 0.2-s recycle delay. Waltz-16 decoupling was employed, and for native dehydrase, 16K data

(42) Parry, R. J.; Ju, S.; Baker, B. J. *J. Labelled Compd. Radiopharm.* **1991**, *29*, 633–643.
(43) Kunitani, M. G. Ph.D. Thesis, Brandeis University, 1977.
(44) Annand, R. R. Ph.D. Thesis, Purdue University, 1992.
(45) Kass, L. R. *Methods Enzymol.* **1969**, *14*, 73–80.
(46) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.

points were acquired over a 32 000-Hz window, giving an acquisition time of 0.267 s. (Unless noted otherwise, the spectral window used for acquisition of data for derivatized dehydrase samples was 30 000 Hz.) Each free induction decay was zero-filled to 32K and processed with 10-Hz line broadening. Other parameters are given in the captions of Figures 3–5.

Generation and Analysis of Chymotryptic Peptides from Photoirradiated, DUO-Inactivated Dehydrase. Dehydrase (55 mg) was inactivated with [6-^{3}H]DUO (2.5 mg; 7.04 μCi) and photoirradiated essentially as described above, except that photoirradiation was conducted in 50-mL Pyrex, screw-capped culture tubes in a turntable photoreactor. The protein solution (44 mg of dehydrase, as determined by liquid scintillation counting of a small aliquot, in 50 mM $(NH_4)_2CO_3$, 8 M urea, pH 8.25) was stirred with dithiothreitol (a 50-fold excess relative to protein cysteine residues; 36 mg) for 1 h at 40 °C. The sample was wrapped in aluminum foil, and iodoacetic acid (64.7 mg; 330 μL of a 196 mg/mL solution in the same buffer) was added. Following an additional 1-h incubation, 50 μL of β-mercaptoethanol was added, and the resulting solution was dialyzed overnight at 4 °C in the dark against 2 × 2 L of 50 mM $(NH_4)_2CO_3$, 2 M urea, pH 8.19. Half of the resulting carboxymethylated, photoirradiated, [6-^{3}H]DUO-inactivated dehydrase (43 mL, 19.5 mg) was adjusted to 11 mM in $CaCl_2$ by the addition of 473 μL of 1.0 M $CaCl_2$. TLCK-treated α-chymotrypsin (500 μL of a 1.74 mg/mL solution in 10 mM HCl) was added to the protein solution and the pH of the solution adjusted to 8.16 with NaOH. Following incubation at 37 °C for 8.5 h, a second 500-μL aliquot of the protease solution was added. The pH of the resulting solution was adjusted to 8.18 using dilute NH_4OH, and the digest was continued for an additional 15 h. A 1-mL aliquot of the chymotrypsin digest solution was clarified by centrifugation at 14 000 rpm in a microfuge for 5 min. (Sixty-seven percent of the radioactive material, corresponding to ca. 0.6 mg of digested protein, remained in solution following centrifugation.) The clarified sample was loaded onto a 25 cm × 4.6 mm Vydac 218TP54 Protein/Peptide C_{18} HPLC column. Following isocratic elution for 10 min with 75% solvent A (water with 0.1% TFA) plus 25% solvent B (5% water, 0.1% TFA in CH_3CN), a 70-min linear gradient to 36.6% solvent B was run, and this was followed by a 5-min linear gradient to 100% solvent B. The flow rate was 1 mL/min, and the effluent was monitored at 220 nm. Fractions (1 mL) were collected and analyzed by liquid scintillation counting. Two additional runs were made, and the peaks corresponding to the radiolabeled peptides were collected manually for analysis by Edman sequencing and FAB-MS.

Generation and Analysis of Tryptic Peptides from Photoirradiated, DUO-Inactivated Dehydrase. A solution (43 mL) of carboxymethylated, photoirradiated, [6-^{3}H]DUO-inactivated dehydrase (19.5 mg) in 50 mM $(NH_4)_2CO_3$, 2 M urea, pH 8.20, was adjusted to 11 mM in $CaCl_2$ by the addition of 473 μL of 1.0 M $CaCl_2$. Diphenylcarbamyl chloride-treated trypsin (218 μL of a 4.43 mg/mL solution in 10 mM HCl) was added to the protein solution, and the pH of the solution was adjusted to 8.16 by the addition of dilute NH_4OH. Following incubation at 37 °C for 8.5 h, a second 218-μL aliquot of the protease solution was added. The pH of the resulting solution was adjusted to 8.10, and the digest was continued for an additional 15 h. A 1-mL aliquot of the digest solution was clarified by centrifugation at 14 000 rpm in a microfuge for 5 min. (Thirty-three percent of the radioactive material, corresponding to ca. 0.3 mg of digested protein, remained in solution following centrifugation.) The clarified solution was loaded onto a 25 cm × 4.6 mm Vydac 218TP54 Protein/Peptide C_{18} HPLC column. Following isocratic elution with 65% solvent A (water with 0.1% TFA) plus 35% solvent B (5% water, 0.1% TFA in CH_3CN) for 10 min, a 50-min linear gradient to 50% solvent B was run, and this was followed by a 10-min linear gradient to 100% solvent B. The flow rate was 1 mL/min, and the effluent was monitored at 220 nm. Fractions (1 mL) were collected and analyzed by liquid scintillation counting. Two additional runs were made, and the peaks corresponding to the radiolabeled peptides were collected manually for sequence analysis and PDMS. A few microliters of each sample was applied to a nitrocellulose-coated mylar target and washed with ca. 50 μL of 0.1% TFA. The dried sample was inserted into the mass spectrometer, and data were collected for 30 min at an acceleration voltage of 17 kV.

Electrospray Ionization Mass Spectrometry. Samples of native dehydrase, DUO-inactivated dehydrase, and photoirradiated, DUO-inactivated dehydrase were desalted on a 25 cm × 4.6 mm Vydac Protein/Peptide C_{18} column and eluted with a 40-min linear gradient from 99.9% water/0.1% TFA to 99.9% CH_3CN/0.1% TFA. Electrospray mass spectroscopic analysis was conducted at the E. I. du Pont de Nemours and Co., Experimental Research Station, Wilmington, DE. Spectra were acquired using a TSQ700 mass spectrometer from Finnigan (San Jose, CA) fitted with a single-stage electrospray ion source from Analytica of Branford. The instrument scanned the range from m/z 500 to 1800 in 3 s. Each spectrum is an average of 10 scans. The samples (10 pmol/μL) were infused at 2 μL/min using a Harvard syringe pump. The spectra were deconvoluted using Biomass algorithms provided by Finnigan and referenced to an external myoglobin standard.

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