SPECTROPHOTOMETRIC DETERMINATION OF MERCAPTIDE ION, AN ACTIVATED FORM OF SH-GROUP IN THIOL ENZYMES

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1. Introduction

The dissociated form of a thiol group, the mercaptide ion, is a far better nucleophile than the nondissociated species [1]. Therefore, the thiol group of a simple SH-compound becomes less reactive as its dissociation is reversed with decreasing pH. On the other hand, in thiol enzymes the reactivity of the essential thiol group often does not parallel the expected dissociation and it may be remarkably high even below the pK_a of thiol group. A possible explanation for this high reactivity is offered by the studies on thiolsubtilisin [2] and papain [3], which indicate that the thiol group of these enzymes exists in the mercaptide ion form even at low pH due to an interaction with a neighboring histidine residue.

In this paper we present a direct method to identify the dissociated form of the SH-group of thiol enzymes. The method is based on measuring the disappearance of *the absorption band of the mercaptide ion* during alkylation. By means of this method in thiolsubtilisin the mercaptide ion could be detected in the pH-range where the mercaptide–imidazolium ion-pair has previously been assumed to exist [2].

2. Experimental

Glutathione (Merck) contained 0.98 mole equivalents of thiol groups as measured with DTNB*/4/.

Mercaptoacetic acid was redistilled twice at reduced pressure under nitrogen (b.p. 104–106°C, 11 mmHg).

Carlsberg thiolsubtilisin was prepared as described previously [5] and was treated with phenylmethanesulfonyl fluoride in order to inhibit the parent serine enzyme present as a small contamination [6].

Chloroacetamide was purified by recrystallization from water. Iodoacetamide and iodoacetic acid were recrystallized from carbon tetrachloride.

3. Results and discussion

The dissociated form of a simple alkyl thiol group can be identified spectrophotometrically since it displays an absorption band around 240 nm with a molar extinction coefficient of about 4000 $M^{-1}cm^{-1}$ whereas the absorption of the protonated form is negligible at and above this wavelength [7]. However, it is difficult to recognize a mercaptide ion in a protein even by conventional difference spectral measurements, as the ionization of other groups, such as tyrosine residues, would mask the spectral change due to SH-dissociation [8].

Our approach to reveal a mercaptide ion in proteins is based on measuring the disappearence of the characteristic absorption band of the mercaptide ion on alkylation at constant pH, rather than on protonation. Thus by measuring the spectra of either an ordinary or an activated SH-group of a protein before and after alkylation, the existence of the mercaptide ion is expected to be demonstrated.

For alkylation of thiolsubtilisin iodoacetamide and iodoacetate were used. Since these alkylating agents and the product, the iodide ion, also have significant absorptions, the observed difference between the optical densities measured before and after

Abbreviation: DTNB, 5,5′-dithiobis (2-nitrobenzoic acid).

Table 1
Correction terms ($\epsilon_{IAAm}{}^{a} - \epsilon_{I^-}$, $\epsilon_{IAAc}{}^{a} - \epsilon_{I^-}$) for calculations of ϵ_{S^-} at different wavelengths.

Wavelength (nm)	$\epsilon_{IAAm}{}^{a}$	$\epsilon_{IAAc}{}^{a}$	ϵ_{I^-}	$\epsilon_{IAAm} - \epsilon_{I^-}$	$\epsilon_{IAAc} - \epsilon_{I^-}$
240	330	320	4 030	−3 700	−3 710
245	301	340	1 680	−1 379	−1 340
250	318	374	583	−265	−209
255	352	400	170	182	230
260	382	408	44	338	364
265	390	400	15	375	385
270	377	371	9	368	362

[a] ϵ_{IAAm} and ϵ_{IAAc} are the molar extinction coefficients of iodoacetamide and iodoacetate, respectively.

the reaction should be corrected according to eq. 1.

$$\epsilon_{S^-} = \Delta\epsilon - (\epsilon_{IAAx} - \epsilon_{I^-}) \quad (1)$$

where $\Delta\epsilon$ stands for the observed change in the absorption during alkylation of 1 mole of thiol group and ϵ_{S^-}, ϵ_{IAAx} and ϵ_{I^-} are the molar extinction coefficients of the mercaptide ion, iodoacetamide or iodoacetate, and the iodide ion, respectively. The correction terms ($\epsilon_{IAAx} - \epsilon_{I^-}$) at different wavelengths, which take into account the changes in absorption due to the decomposition of iodoacetamide or iodoacetate ion and the formation of iodide ion, are shown in table 1. It is seen from the table that the sign of the correction term depends on the wavelength. The correction term is zero at 252 nm with iodoacetamide and at 251.5 nm with iodoacetate ion. If chloroacetamide or chloroacetate is used as alkylating agent, the correction terms can be neglected relative to the molar absorptivity of the mercaptide ion in the wavelength range of 245–270 nm. However, in most cases it is more convenient to use the iodocompounds since they react much faster with the thiol group than do the chloroderivatives.

First we have studied the change in absorbance of glutathione, a simple thiol compound, during alkylation by iodoacetamide at different wavelengths. The reactions were followed at 25°C under pseudo first-order conditions with excess iodoacetamide at pH 10.8, where the thiol group is practically in the dissociated form ($pK_a \sim 9$). The rate constans measured at any wavelength between 245 and 265 nm agreed within experimental error (27 M^{-1} sec^{-1}) and were consistent with those determined previously by the DTNB method (30 M^{-1} sec^{-1} in the presence of 1 M KCl, [2]). The absorption of the reaction mixture at zero time

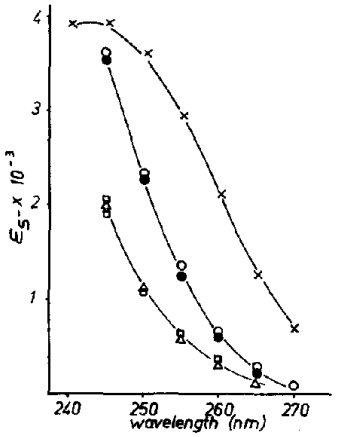

Fig. 1. Spectra of glutathione and mercaptoacetate, and ϵ_{S^-} values of glutathione and thiolsubtilisin. The spectrum of glutathione (○–○–○) was measured in 0.1 M carbonate buffer, pH 10.8. The spectrum of mercaptoacetate (X–X–X) was determined in 0.01 M NaOH. ϵ_{S^-} values for glutathione (●–●–●) were calculated from the reactions of 5.7×10^{-5} M glutathione with 98×10^{-5} M iodoacetamide at 25.0°C in 0.1 M carbonate buffer, pH 10.8. ϵ_{S^-} values for thiolsubtilisin were calculated from the reaction of 4.4×10^{-5} M thiolsubtilisin type Carlsberg with 107×10^{-5} M iodoacetamide (△–△–△) or 174×10^{-5} M iodoacetate (□–□–□) at 25.0°C in 0.1 M phosphate buffer, pH 7.0.

was determined by extrapolating the pseudo first-order progress curve. Fig. 1 shows that the ϵ_{S^-} values calculated from alkylation of glutathione in the range of 245–265 nm conform to the actual spectrum of the thiol compound within experimental error. This justifies the use of alkylation to determine the mercaptide ion.

In the case of proteins, it is more difficult to mea-

sure the change in the optical density on alkylation of the thiol group because the absorptivity of the mercaptide ion is much lower than that of the rest of the protein. The molar absorptivity of subtilisin type Carlsberg is 26 700 $M^{-1}cm^{-1}$ at 280 nm [9]. With decreasing wavelength the absorption of the protein decreases down to 250 nm, whereas that of the mercaptide ion increases. The optimal wavelengths for monitoring alkylation are at 250–255 nm where the absorption of the protein is only one order of magnitude higher than that of mercaptide ion. Using the expanded scale (0.0–0.1) of an Opton DMR 21 recording spectrophotometer fitted with a thermostated cell compartment, we could readily measure 1% change in absorbance. The high absorption of the reaction mixture was compensated for in the reference cell by appropriate concentrations of sodium cinnamate in phosphate buffer, pH 7.0. The alkylation of thiolsubtilisin was carried out under pseudo first-order conditions at 25.0°C as described above for glutathione. The curves obtained for thiolsubtilisin, reacted with either iodoacetamide or iodoacetate at pH 7.0, resemble that found with the dissociated thiol group of glutathione (fig. 1). The somewhat lower values can be attributed to the interaction between the mercaptide and the imidazolium ions which results in the formation of an ion-pair in the range of pH 6–8.5 [2]. It is noteworthy that the negative carboxylate ion of mercaptoacetate causes an opposite shift relative to the spectrum of glutathione as does the positive imidazolium ion (fig. 1).

In agreement with the idea of the ion-pair, in thiolsubtilisin the ϵ_S-, or rather ϵ_{IP}, the molar extinction coefficient of the mercaptide ion in the ion-pair, does not change significantly between pH 6 and 8.5 (fig. 2). Unfortunately at higher pH's, where the free mercaptide ion exists, we could not determine ϵ_S- because the absorption of the protein strongly increases with pH. On the other hand, the apparent ϵ_S- values of glutathione, which approximate the actual ϵ_S- at high pH, fit to a normal dissociation curve, which proves again that we only measure the dissociated form of the thiol group. It may be noted that the experimental points measured with both iodoacetamide and chloroacetamide conform to the same curve (fig. 2).

The above experimental data suggest that the corrected spectral changes during alkylation of thiolsubtilisin are due to the disappearance of the characteristic absorption band of the mercaptide ion. The observed spectral changes are not markedly affected by alkylation of other groups and conformational changes in the protein as indicated by the following facts. The mixture of subtilisin, the parent serine enzyme, and iodoacetamide shows no spectral changes under the conditions used for alkylation of thiolsubtilisin. Accordingly, even if conformational changes do occur they must be connected with alkylation of the thiol group. Nevertheless, the only chromophore that can be directly affected by the carboxamidomethyl or carboxymethyl group bound covalently to the thiol group is the neighboring histidine residue as shown by the steric structure of subtilisin [10]. However, the change in the absorption of an imidazole chromophore on protonation is about one tenth of the corresponding change for the sulfhydryl group [11]. Furthermore, the change in absorption during alkylation of thiolsubtilisin is independent of the fact whether alkylation was performed with iodoacetamide or iodoacetate (fig. 1). It is rather improbable that a neutral and a negatively charged alkylating agent would cause identical structural changes.

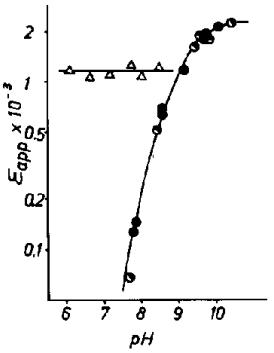

Fig. 2. The pH-dependence of the apparent molar absorptivity of glutathione and thiolsubtilisin as measured by alkylation. Thiolsubtilisin was reacted with iodoacetamide (△–△–△), glutathione with iodoacetamide (●–●–●) and chloroacetamide (◑–◑–◑) at 250 nm. Reaction conditions were similar to those in Fig. 1, except at low molar absorptivities of glutathione, where the concentration of the reactants were increased up to 10-fold. The concentration of chloroacetamide was (4.48–13.44) $\times 10^{-2}$ M.

Our previous study on thiolsubtilisin [2] has indicated that the ion-pair is the reactive nucleophile of the enzyme, but a possible equilibrium between the thiolate–imidazolium and the thiol–imidazole

systems could not be demonstrated. By the present spectrophotometric method, we could show that the SH-group of thiolsubtilisin is mainly present as a mercaptide ion in the pH-range of 6–8.5, which implies that the equilibrium is shifted to the formation of the ion pair.

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