Spectrophotometric Measurement of the Liberation of the α -Amino Group of Cysteine Residues in Polypeptides

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1. The reaction between β -bromopyruvic acid and SH groups of cysteine residues in reduced ribonuclease and in some other polypeptides was investigated. 2. One molecule of the acid was found to be necessary to block one SH group in reduced ribonuclease. The stoichiometry of the interaction and the spectral characteristics of the compound formed suggested that the product is an *S*-oxalomethyl ($R \cdot S \cdot CH_2 \cdot CO \cdot CO_2H$) derivative of reduced ribonuclease. 3. Digestion of reduced *S*-oxalomethylated ribonuclease by trypsin or chymotrypsin induced changes in the spectrum that could be attributed to the liberation of the α -amino group of *S*-oxalomethylated cysteine residues from peptide bonds. The spectral changes that accompanied the hydrolysis of specific peptide bonds in reduced *S*-oxalomethylated ribonuclease and *S*-oxalomethylated co-poly(L-Lys, L-CySH) allowed the kinetics of the digestion to be followed. 4. Possible applications of the spectrophotometric method in the study of protein structure are discussed.

It has been shown that in the reaction between thiol compounds and β -halopyruvic acids substitution of the SH group occurs, and *S*-oxalomethylated derivatives ($R \cdot S \cdot CH_2 \cdot CO \cdot CO_2H$) are formed (Avi-Dor & Mager, 1956; Bergmann & Mielewicz, 1963).

The difference spectrum, obtained by subtracting from the spectrum of the product that of the reactants, exhibits a characteristic band in the ultraviolet region with a peak at about 270 m μ (type I spectrum). With thiol compounds possessing an unsubstituted amino group in the position α or β to the thiol group, the peak is shifted to 300 m μ and the molar extinction is increased nearly tenfold (type II spectrum). The latter bathochromic and hyperchromic effect is presumably connected with formation of a Schiff base between the carbonyl group of oxalomethyl residue and the amino group of the amino thiol (cf. Strukov, 1958; Hermann, 1961; Bergmann & Mielewicz, 1963). *S*-Oxalomethylated derivatives of *N*-substituted amino thiols, such as *S*-oxalomethylated GSH, gave the type I spectrum. During hydrolysis of the peptide bond, in which the α -amino group of *S*-oxalomethylated cysteine is involved, the difference spectrum changes from type I to type II (Avi-Dor, 1960).

In the present investigation the reaction between β -bromopyruvic acid and SH groups of reduced ribonuclease and of other polypeptides was studied. As with GSH, it was found that the enzymic hydrolysis of peptide bonds in which the α -amino group of an *S*-oxalomethylated cysteine residue is

involved is accompanied by an increase in the extinction at 300 m μ . The change in the spectrum allows the kinetics of the hydrolysis of the peptide bond to be followed spectrophotometrically.

MATERIALS AND METHODS

Materials. Ribonuclease A, yeast alcohol dehydrogenase, trypsin (type I), chymotrypsin and thiolated gelatin (type II) were products from Sigma Chemical Co., St Louis, Mo., U.S.A. The synthetic polypeptides were a generous gift from Professor A. Patchornik of the Weizmann Institute of Science, Rehovoth, and were synthesized by Yeda Research and Development Co., Rehovoth, Israel. Co-polymers of cysteine were obtained in the form of *S*-benzyloxycarbonyl derivatives. To liberate the SH groups, the co-polymers were dissolved in a minimum amount of dimethylformamide. To this solution 5 equiv. (with respect to cysteine content) of sodium methoxide was added. At room temperature the hydrolysis was complete in 5 min. The product of hydrolysis was acidified with acetic acid and used on the same day. Reduced ribonuclease was prepared according to the method of Epstein, Goldberger, Young & Anfinsen (1962). β -Bromopyruvic acid was obtained by bromination of freshly distilled pyruvic acid under the conditions described by Dickens & Williamson (1958) with the modifications that the pyruvic acid and the bromine were dissolved in carbon tetrachloride and the recrystallization of the products was carried out from benzene.

Methods. Spectrophotometric titration of SH groups was carried out according to the method of Boyer (1954), with *p*-chloromercuribenzoic acid as the reagent. Spectrophotometric measurements were conducted in a Zeiss PMQII spectrophotometer except for the kinetic experiments presented in Figs. 5 and 6, which were carried out in a

Beckman spectrophotometer (model DP) equipped with a Linear and Log Potentiometric Recorder. Silica cells of 1 cm. light-path were used.

RESULTS

Interaction of β -bromopyruvic acid with SH groups of reduced ribonuclease. Ribonuclease A was chosen as a model compound for the study of the interaction between β -bromopyruvic acid and polypeptides because of its well established primary structure (Hirs, Stein & Moore, 1956; Smyth, Stein & Moore, 1963). When reduced ribonuclease (7.2–8.0 SH groups/molecule) is treated at room temperature with an excess of β -bromopyruvic acid, no SH group can be detected with *p*-chloromercuribenzoate after 3–5 min. Fig. 1 shows the difference spectrum obtained by subtracting the spectrum of the reactants from that of the products. For comparison the spectrum of *S*-oxalomethylated GSH is also given. The similarity between the two spectra is striking. It should be noted that to get reproducible results it is necessary to add EDTA to the reaction mixture, presumably because traces of metal impurity may change the location and the height of the peak (see below).

The close relation between the product derived from reduced ribonuclease and that obtained from

Fig. 1. Difference spectra of the spectrum of reduced *S*-oxalomethylated ribonuclease minus that of reduced ribonuclease (●) and of the spectrum *S*-oxalomethylated GSH minus that of GSH (○). The interaction between β -bromopyruvic acid and the thiol compounds was carried out at room temperature in 0.30 M-tris buffer, pH 7.4, containing EDTA (2 mM). The concentration of the thiol compounds was 0.10 mM with respect to SH content and that of β -bromopyruvic acid was 0.8 mM. The spectrum was taken after the increase in $E_{270\text{ m}\mu}$ reached its maximum (3–5 min.).

GSH is also revealed by the experiments shown in Fig. 2. These experiments are based on a previous observation, according to which the difference spectrum obtained with *S*-oxalomethylated derivatives is changed from type I to type II in the presence of borate (Avi-Dor & Lipkin, 1958) or under the influence of certain metal ions (Avi-Dor, 1959). As seen from Fig. 2, when the interaction between β -bromopyruvic acid and either GSH or reduced ribonuclease is conducted in borate buffer or when to the products of the reaction obtained in tris buffer Al^{3+} ions are added, the absorption band in the difference spectrum shifts towards the longer wavelength and is greatly intensified (Al^{3+} and Zn^{2+} ions were equally effective on *S*-oxalomethylated GSH but Al^{3+} acted at much lower concentration on the compound derived from reduced ribonuclease).

Specificity and stoichiometry. The experiments summarized in Table 1 were carried out to test whether the characteristic band in the ultraviolet region that appears when β -bromopyruvic acid reacts with reduced ribonuclease is specifically connected with the substitution of SH groups. β -Bromopyruvic acid was allowed to act on various polypeptides, some of them containing free SH groups, such as reduced ribonuclease (Hirs, Moore & Stein, 1960), thiolated gelatin (Benesch & Benesch, 1958), alcohol dehydrogenase (Van Eys, Ciotti & Kaplan, 1957), co-poly(L-Lys, L-CySH) and co-poly(DL-Ala, L-CySH), others possessing the sulphur atom in a disulphide linkage (native ribonuclease) or lacking thiol groups altogether [poly(L-Lys)]. An inspection of the results reveals that the interaction between β -bromopyruvic acid and polypeptides results in an increment in the extinction only when the polypeptide contains free SH groups.

The stoichiometry of the reaction was ascertained by adding known amounts of β -bromopyruvic acid to reduced ribonuclease and determining the number of unchanged SH groups with *p*-chloromercuribenzoate. From Table 2 it is apparent that each molecule of β -bromopyruvic acid blocked one SH group, thus suggesting that under the conditions used no reaction other than substitution of the SH groups is taking place.

Effect of proteolytic enzymes. The results described above suggested that, whether β -bromopyruvic acid acts on SH groups of polypeptides or on thiol compounds of low molecular weight, analogous products are formed. It could thus be expected that hydrolysis of the peptide bond in which the α -amino group of an *S*-oxalomethylated cysteine residue is involved should lead to a change in the spectrum from type I to type II and, as corollary, to an increase in $E_{300\text{ m}\mu}$. This assumption was verified in the experiments shown in Figs. 3–5.

Fig. 2. Effect of borate and Al^{3+} ions on the spectrum of the product of interaction of β -bromopyruvic acid with reduced ribonuclease (a) and with GSH (b). The difference spectra $\circ$ and $\bullet$ were measured in 0.3 M-tris buffer containing EDTA (2 mM); the difference spectra $\triangle$ and $\blacktriangle$ were measured after incubating reduced ribonuclease and GSH with β -bromopyruvic acid in 0.2 M-borate buffer, pH 8; the difference spectra $\square$ and $\blacksquare$ were obtained when the product of reaction in 0.3 M-tris buffer not containing EDTA was incubated with 0.1 mM- $\text{Al}_2(\text{SO}_4)_3$ for 20 min. All other conditions were as given in Fig. 1.

Table 1. *Increases in $E_{270\text{ m}\mu}$ as a result of the action of β -bromopyruvic acid on various polypeptides*

The conditions for the reaction were as given in Fig. 1. The number of SH groups/molecule of substance (or the concentration of SH groups in the reaction mixture, when the molecular weight was unknown) was determined with *p*-chloromercuribenzoate.

Substance	$E_{270\text{ m}\mu}$ (per mole of SH)	No. of SH groups/molecule	Concn. of SH groups (mM)
Ribonuclease	None	None	—
Alcohol dehydrogenase	600–700	13–14	—
Reduced ribonuclease	500–700	7.2–8.0	—
Co-poly(L-Lys,L-CySH)	600	—	0.10
Co-poly(L-Lys,L-CySH)	600	—	0.10
Co-poly(DL-Ala ₂₀ ,L-CySH)	700	—	0.12
Thiolated gelatin	700	—	0.14
Poly(L-Lys)	None	None	—

The proteolytic enzymes trypsin and chymotrypsin were used for the digestion of the product of interaction of β -bromopyruvic acid and reduced ribonuclease. The action of the above enzymes has

already been studied in great detail on reduced *S*-carboxymethylated ribonuclease by Crestfield, Moore & Stein (1963) and on oxidized ribonuclease by Hirs *et al.* (1960). Fig. 3 shows that, on adding

Table 2. Titration of reduced ribonuclease with *p*-chloromercuribenzoate after pretreatment with various amounts of β -bromopyruvic acid

The preincubation with β -bromopyruvic acid was carried out in 0.3 M-tris buffer, pH 8, at room temperature for 15 min. The concentration of the reduced ribonuclease was 0.06 mM with respect to SH content. β -Bromopyruvic acid was added as indicated in the Table. The number of SH groups that remained after the reaction with β -bromopyruvic acid was determined with *p*-chloromercuribenzoate, as described in the Materials and Methods section.

Equiv. of β -bromopyruvic acid/mol. of reduced ribonuclease	Unchanged SH groups/mol. of reduced ribonuclease
None	8.3
2.4	5.6
4	4.2
5.3	2.8
8	None

Fig. 3. Time-course of the increase in $E_{300\text{ m}\mu}$ on digesting reduced *S*-oxalomethylated ribonuclease with chymotrypsin (▲) or trypsin (●). The interaction between reduced ribonuclease (0.36 mM with respect to SH content) and β -bromopyruvic acid (2 mM) was carried out at room temperature in 0.3 M-tris buffer, pH 8, containing EDTA (2 mM). A sample of the product was digested at room temperature with 0.02 mg. of the proteolytic enzyme/ml.

either trypsin or chymotrypsin to reduced *S*-oxalomethylated ribonuclease, a continuous rise is observed in $E_{300\text{ m}\mu}$. The total change in $E_{300\text{ m}\mu}$ is larger with chymotrypsin than with trypsin. In the experiment shown in Fig. 4 the digestion was started by adding trypsin and then repeating the addition after $E_{300\text{ m}\mu}$ had reached a plateau: it can be seen that no further increase in $E_{300\text{ m}\mu}$ takes place. When, however, chymotrypsin is added at this stage, the rise in $E_{300\text{ m}\mu}$ is renewed and terminates only

Fig. 4. Digestion of reduced *S*-oxalomethylated ribonuclease by adding trypsin and chymotrypsin in sequence to the same sample of the substrate. The digestion was started by adding 0.02 mg. of trypsin/ml. of the reaction mixture. At the time indicated by the first arrow (A) the addition of trypsin was repeated. The second arrow (B) denotes the time of addition of chymotrypsin. Other conditions were as given in Fig. 3.

Fig. 5. Difference spectrum of the spectrum of reduced *S*-oxalomethylated ribonuclease after digestion by trypsin minus that of the reactants before digestion (▲). Conditions for the formation of reduced *S*-oxalomethylated ribonuclease and for the digestion were as given in Fig. 3. Spectrum of the product formed in interaction of 0.05 mM-cysteine with 0.4 mM- β -bromopyruvic acid in 0.3 M-tris buffer, pH 7.4 (●).

when the total increment in $E_{300\text{ m}\mu}$ approximates the sum of the effects obtained on adding each of the two enzymes to different samples of the

Fig. 6. Time-course of the increase of $E_{300\text{ m}\mu}$ on digesting *S*-oxalomethylated co-poly(L-Lys, L-CySH) by trypsin (0.02 mg./ml.). The reaction between the co-polymer (0.10 mM with respect to SH groups) and β -bromopyruvic acid (0.8 mM) was carried out in 0.5 M-phosphate buffer, pH 7.4, containing EDTA (3 mM).

substrate. By subtracting the spectrum of the undigested compound from that of the products of digestion, a difference spectrum is obtained with a peak at 300 m μ . The similarity between this difference spectrum and the one obtained by interaction of β -bromopyruvic acid and cysteine is obvious (Fig. 5).

Effect of trypsin on S-oxalomethylated co-poly(L-Lys, L-CySH). The sequence Lys-CySH, which is subject to attack by trypsin, must occur with great frequency in co-poly(L-Lys, L-CySH). The hydrolysis of this bond in the *S*-oxalomethylated co-polymer should result in a spectral change analogous to that described in the preceding paragraph. The experimental findings are in accord with this expectation since when trypsin is added to *S*-oxalomethylated co-poly(L-Lys, L-LySH) it causes a gradual increase in $E_{300\text{ m}\mu}$ (Fig. 6).

DISCUSSION

The present results are in accord with the view that the spectral changes noted during the interaction of β -bromopyruvic acid with reduced ribonuclease and other cysteine-containing polypeptides are due to the substitution of SH groups by the reagent. If only non-terminal cysteine residues are present, the product gives the same difference spectrum as the *S*-oxalomethylated derivatives of simple thiol compounds of low molecular weight (i.e. λ_{max} . 265–270 m μ ; ϵ_{max} . 600–700). *N*-Terminal cysteine residues, on the other hand, form compounds with β -bromopyruvic acid the spectra of which very closely resemble that of the products derived from α -amino thiols (λ_{max} . 300 m μ). Hence,

when during enzymic digestion of a polypeptide a non-terminal *S*-oxalomethylated cysteine residue is converted into a terminal one, the simultaneous change in the spectrum permits the time-course of the hydrolysis to be followed.

If we assume that the molar extinction of the compound from *N*-terminal cysteine residue is the same as that obtained from free cysteine (i.e. 5000 at 300 m μ), then the number of X·CO·NH-(CyS·R) peptide bonds split can be computed. A calculation from the data presented in Fig. 3 shows that trypsin hydrolyses 0.70 and chymotrypsin 0.90 X·CO·NH-(CyS·R) peptide bond/molecule of reduced ribonuclease. Since the sum of the increments in $E_{300\text{ m}\mu}$ was nearly the same when the above two enzymes acted in sequence on the same sample of the substrate as when they acted separately on different samples, the bonds that are attacked by trypsin and chymotrypsin respectively cannot be identical. The known amino acid sequence in ribonuclease (Smyth *et al.* 1963) and the data published in the literature on the action of proteolytic enzymes on reduced carboxymethylated ribonuclease (Crestfield *et al.* 1963) allows us to identify the peptide bonds the hydrolysis of which is responsible for the spectral changes. They are the bonds between tyrosine (no. 25) and cysteine (no. 26) and that between arginine (no. 39) and cysteine (no. 40), which are hydrolysed by chymotrypsin and trypsin respectively. It is conceivable that by using a similar approach to that described above one could also estimate the number of basic amino acid-cysteine sequences in proteins of unknown primary structure.

Another application of the spectrophotometric assay may be in connexion with studies on controlled reduction and reoxidation of disulphide bonds in proteins. For example, by converting the partially reduced ribonuclease into its *S*-oxalomethylated derivative and subjecting it to digestion by trypsin and chymotrypsin, one could determine whether the cysteine residues nos. 26 and 40 possess free thiol groups or are linked in a disulphide bond.

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