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MICROHETEROGENEITY IN PORCINE PANCREATIC AMYLASE PREPARATIONS DUE TO DISULFIDE-SULFHYDRYL EXCHANGE

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Summary

There are two masked SH groups in pancreatic amylase (EC 3.2.1.1) which become reactive after removal of Ca ions, and then only due to fluctuation of the polypeptide chain. Carboxymethylation of the masked SH groups and isolation of the tryptic CM-peptides have shown that the reacting SH groups in active amylase preparations are not identical, depending on the method of preparation. The microheterogeneity is attributed to an SH-SS exchange reaction taking place during preparation. This exchange results in a mixture of two types of amylase molecules containing different SH groups.

The tryptic peptides containing the SH groups have been detected from the radioautogram of the peptide map of the [^{14}C]carboxymethylated protein digest. We identified the SH peptides present in native amylase, and those cysteinyl peptides which form an easily reducible disulfide in the vicinity of these thiol groups, and take part in the intramolecular SH-disulfide exchange.

Introduction

Pancreatic amylase (1.4 D-glucan-glucanohydrolase, EC 3.2.1.1) is a single-chain polypeptide and has a molecular weight of 52 000 [1,2]. It contains 2 SH groups and 4 disulfide bonds per molecule. The SH groups are unreactive in the native enzyme but react in the presence of EDTA or concentrated urea [3]. We have shown that the rate of reaction of amylase with Nbs_2, in the presence of EDTA, depends on the fluctuation of the protein structure [4]. Moreover, we gave evidence that amylase, when its SH groups are completely blocked

Abbreviations used: 5.5 dithio-bis (2-nitrobenzoate), Nbs_2 carboxymethyl-, CM-.

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with Nbs_2, is enzymatically fully active [4]. This was confirmed by others [5, 6]. The formation of an internal disulfide from the two buried SH groups under certain conditions was shown, and explained by assuming that these SH groups are close to each other within the structure of the enzyme [4]. One of the disulfide bridges is more easily reduced than the others [4,7], again without loss of enzyme activity. This disulfide is reformed in a dilute neutral solution (due to oxidation of SH by dissolved oxygen.

The ten tryptic cysteinyl peptides of fully reduced amylase have been isolated and characterized earlier [8]. The aim of the present work was to identify the SH groups of amylase and the cysteinyl residues involved in the formation of the easily reducible disulfide bridge.

Materials and Methods

Soluble starch was purchased from Merck (G.F.R.) and Reanal (Hungary). Nbs_2 was a Fluka (Switzerland) product, 10 mM stock solution (pH 7.5) was used. Area (A grade) was from Reanal (Hungary) and recrystallized from 70% ethanol. Dithiothreitol and phenylmethylsulfonylfluoride were Serva (G.F.R.) preparations. [^{14}C]bromoacetate was obtained from the Institute of Isotopes (Hungary) and it had a specific activity of 1.8—2.4 Ci per mol. Trypsin, essentially free of chymotrypsin, was Calbiochem (U.S.A.) preparation. Diisopropylfluorophosphate was obtained from Boots Pure Drug Co. Ltd. (Great Britain). Sephadex G-25, G-50 and DEAE-Sephadex A-50 were products of Pharmacia (Sweden).

Amylase activity was determined at 37° C with 0.2 μg per ml amylase in a 10-ml reaction volume according to Smith and Roe [9], by the iodine-starch method, measured at 620 nm.

SH groups of amylase were determined with Nbs_2, according to Ellman's method [10], measuring the absorption at 412 nm, in 0.1 M Tris · HCl buffer, pH 8.5 in the presence of 3 mM EDTA, or in 8 M urea containing EDTA.

Enzyme preparations

Pancreatic amylase from pig pancreas was prepared by two, somewhat different, methods.

Method 1. The producedure of Hatfaludi et al [11] was applied as described.

Method 2. The above procedure was modified so that treatment with concentrated urea, for the purpose of recrystallisation, was eliminated. Instead, the crystals obtained from the first crystallization were washed with cold water. The preparation obtained has the same specific activity as that obtained by Method 1.

The modified procedure was as follows:

1. Pig pancreas freed of gross fat and connective tissue was homogenized three times in a mixer for 1 min every time, with 10 mM $CaCl_2$ solution containing 3% butanol (portions of 150 g pancreas were homogenized with 100 ml of this mixture). The resulting pulp was warmed in a waterbath to 37° C (this took about 30 min) and when this temperature was reached it was kept at 37° C for another 30 min. After cooling to + 10° C, cold acetone (—10° C) was added with constant stirring (1 l acetone per 1.5 l of extract). The mixture was placed in a

bag of tissue cloth and placed over a funnel of appropriate size to drain off overnight in a cold room, without the application of outside pressure.

2. The clear pale yellow fluid was mixed with a second lot of acetone (818 ml acetone per 1 l fluid), when amylase precipitated as a rapidly settling sticky white material. Most of the supernatant could be decanted and the precipitate was collected in a refrigerated centrifuge. The tubes were well drained of acetone and the precipitate suspended in 10 vols. of a 10 mM $CaCl_2$ solution. This was dialysed overnight against a great excess of a 1 mM $CaCl_2$ solution in the cold.

3. The dialysed solution was centrifuged the following morning to remove denatured proteins. To every liter supernatant, 0.2 ml of an 0.5% phenylmethylsulfonylfluoride solution (in dioxane) was added; then it was left standing in the icebox. Crystallization of amylase took about 10 days, but could be accelerated by seeding with amylase crystals.

4. The crystals were sedimented in the cold at low speed, then washed 5 times with 2—3 vols. of cold 10 mM $CaCl_2$ solution, which should have a pH around 6. Each time the crystals were taken up with fresh $CaCl_2$ solution, they were stirred for 1 min and centrifuged again. Amylase crystals were poorly and slowly dissolved in cold water below pH 7, but contaminating material was completely eliminated by the washing procedure. The crystals could be kept suspended in 10 mM $CaCl_2$ solution for months in the cold.

The specific activity of preparations varied between 5500 and 6000 units per mg protein determined according to Smith and Roe [9].

Protein concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of $A^{1\%}_{1cm} = 24$ [12].

Amylase I and II were separated by chromatography on DEAE-Sephadex A-50 column, as described by Straub et al. [13].

Determination of all tryptic cysteinyl peptides from fully reduced amylase

A 20 mg amylase solution in 5 ml urea 8 M, pH 8.5, was incubated with 2.5 mol of dithiothreitol per mol of half cystine at room temperature for 4 h. A 5-fold molar excess of [^{14}C] bromoacetate was then added, over the total SH present. After 2 h incubation at room temperature the excess of bromoacetate was removed with 2-mercaptoethanol and the protein was desalted by dialysis against 10 mM HCl.

Digestion of the protein with trypsin was performed in 1% $NH_4(HCO_3)$, pH 8.0, for 4 h at 37° C. The trypsin : amylase ratio was 1 : 40 by weight. Digestion was stopped by boiling or by excess diisopropyl-fluorophosphate, and the digest was freeze-fried.

Fingerprints of tryptic digests were prepared by two dimensional electrophoresis [8]: in the first dimension the paper was moistened with pH 6.5 buffer (pyridine/acetic acid/water, 100 : 4 : 896) and pH 5.0 buffer (pyridine/acetic acid/water, 10 : 10 : 980) [14] and in the second dimension pH 1.9 buffer (formic acid/acetic acid/water, 20 : 80 : 900) was used.

To isolate and analyse the cysteinyl peptides, the digest was subjected to gel-filtration on a 2 × 180 cm Sephadex G-25 (fine) column with 0.1 M $NH_4(HCO_3)$, pH 8.2 as eluent. The radioactive fractions were purified by paper electrophoresis and paper chromatography in the buffers and solvents previous-

ly applied [8]. The N-terminal sequences of the radioactive peptides were determined by the dansyl-Edman method [15], the dansyl amino acids were identified by thin-layer electrophoresis [16] or by chromatography on polyamide thin-layer sheets [15]; C-terminal sequences were determined by a micro method [17] using ion-exchange resin coated chromatoplates (Ionex-25 SA, Macherey-Nagel and Co., Düren) for the identification of the amino acids released [18].

The homogeneous peptides were hydrolysed with 20% HCl at 105° C for 24 h.

Amino acid analysis was carried out according to the single column procedure of Dévényi [19] on a Beckman-Unichrom analyzer.

Identification of tryptic peptides containing the SH groups of amylase

To a 2-ml solution containing 10 mg amylase, 10 mM EDTA, 40 mM Tris · HCl buffer, pH 8.5 [^{14}C]bromoacetate was added (bromoacetate: SH molar ratio, 15 : 1). After 2 h incubation at room temperature, the protein was precipitated and washed with trichloroacetic acid, final concentration 5%, until no radioactivity could be detected in the supernatant.

The precipitated protein was redissolved in 8 M urea containing 40 mM Tris · HCl buffer, pH 8.5, and 2.5 mol of dithiothreitol per mol of half-cystine were added and incubated for 2 h at room temperature. After this reduction of disulfide bonds, unlabelled bromoacetate was added in a 15 : 1 molar ratio in excess of SH groups present. After 2 h incubation at room temperature, the excess reagent was removed, the protein desalted and further processed by tryptic digestion and fingerprinting as described above.

When the radioactivity of the individual tryptic peptides was determined, the radioactive spots were identified by radioautography (High Sensitivity X-ray

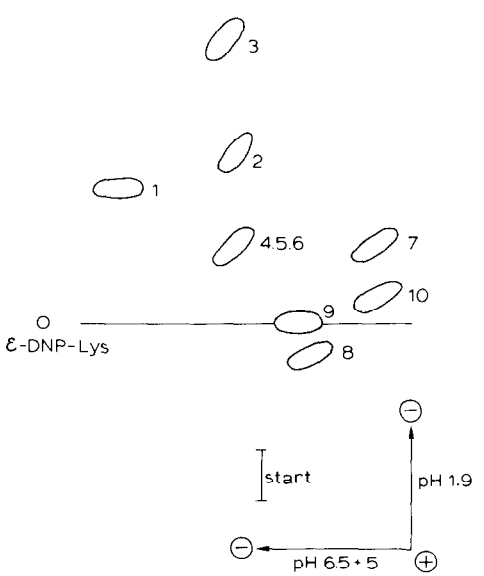


Fig. 1. Radioautogram of [^{14}C]CM-Cysteinyl peptides of tryptic digest of fully reduced amylase. Experimental conditions: see Materials and Methods.

Film, Forte, Hungary, was used); after cutting out or eluting the corresponding peptide spot with 50 mM NH_4OH, radioactivity was measured in a Packard Tricarb scintillation spectrometer, type 2420.

Results

All the 10 CM-cysteinyl peptides from the tryptic digest of fully reduced and [^{14}C] carboxymethylated amylase have been identified by one of us previously [8]. The results of this work are summarized in Fig. 1 and Table I.

It was the aim of the present work to determine which of the cysteinyl residues represent the two SH groups of amylase. In the presence of EDTA, when these 2 SH groups would react with SH reagents, we carboxymethylated the enzyme with [^{14}C] bromoacetate. Then the protein was fully reduced and reacted with non radioactive bromoacetate (cf. Materials and Methods). So we obtained a partially labelled, but fully carboxymethylated enzyme. The radioactivity of the spots on the tryptic fingerprint was correlated with the peptide spots (Figs. 2 and 3).

In Table II results are given in percent of the sum of radioactivity found over the spots of cysteinyl peptides. It has been shown earlier that spot 4—5—6 is a mixture of 3 CM-cysteinyl peptides, which can be resolved by paper-chromatography [8]. In the samples which had been partially labelled with [^{14}C] bromoacetate, only one of them, peptide 4, was found to contain the labelled CM-group. Therefore radioactivity found over the peptide spot 4—5—6 is regarded as the measure of label in peptide 4.

Significantly, we have obtained different results according to the method of preparation. Typical autoradiograms are shown in Figs. 2 and 3, the former being the result obtained in an experiment for which amylase was prepared by the modified method (Method 2, see Materials and Methods) whereas the result shown in Fig. 3 was obtained when amylase was prepared by Method 1 [11].

We believe that the picture obtained using amylase prepared according to Method 2 reflects the structure of native amylase, whereas the results obtained

TABLE I

AMINO ACID SEQUENCES OF THE TRYPTIC CM-CYSTEINYL PEPTIDES OF AMYLASE

See ref. 8.

Number of peptide (see Fig. 1)	Sequence
1.	Val.Ser.Ser.CM-Cys.Tyr.Arg.
2.	Leu.CM-Cys.Thr.Thr.Arg
3.	CM-Cys.Lys
4.	CM-Cys.Asn.Asn.Val.Gly.Val.Arg.
5.	Val.Gly.Asn./CM-Cys_1, Thr_1, Ser_1, Gly_{0-1}/.Ile.Lys
6.	CM-Cys.Asn.Val.Thr.Arg
7.	CM-Cys.Asp.Val.Ile.Ser.Gly.Lys
8.	Asx./CM-Cys_1, Asx_1, Thr_{1-2}, Ser_1, Gly_5, Ala_2, Trp_x/.Tyr.Leu.Arg
9.	Gly/CM-Cys_1, His_1, Tyr_x, Leu_{1-2}, Ile_{1-2}, Val_{0-1}, Gly_1, Ala_1, Thr_1, Ser_1, Asx_2/.Lys
10. *	Ala.Asx./CM-Cys_1, Glx_1, Asx_1/

* Tryptic peptide further digested with pepsin.

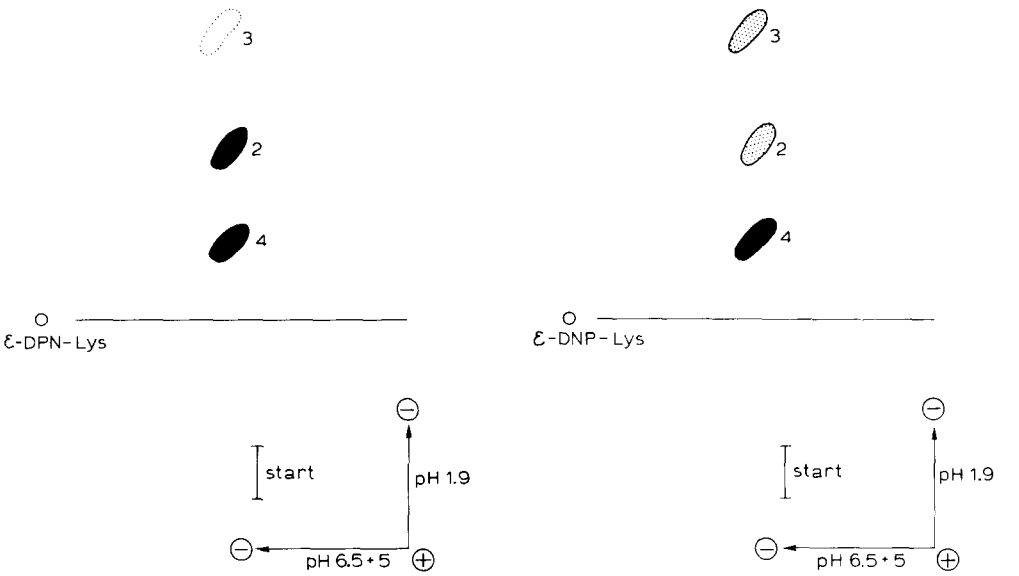


Fig. 2. Radioautogram of [^{14}C]CM-Cysteinyl peptides of tryptic hydrolysate of amylase prepared by Method 2. Experimental conditions: see Materials and Methods.

Fig. 3. Radioautogram of [^{14}]CM-Cysteinyl peptides of tryptic hydrolysate of amylase prepared by Method 1. Experimental conditions: as in Materials and Methods.

with an enzyme prepared according to the original method of Hafaludi et al. is the result of the treatment of the enzyme with concentrated urea used for recrystallization.

This could easily be shown to be the case, when an enzyme, prepared according

TABLE II

DISTRIBUTION OF RADIOACTIVITY OF ^{14}C-LABELED CARBOXYMETHYLCYSTEINYL GROUPS OVER PEPTIDE SPOTS OF THE TRYPTIC FINGERPRINTS OF PANCREATIC AMYLASE

Method of amylase preparation	Treatments of amylase	Percent of total radioactivity (average) over spot number:				mol SH titrated	Number of preparations
		4	2	3	9	mol amylase	
1 Method 1		45	27	26	0	2	19
2 Method 2		46	45	0	0	2	6
3 Method 2	Urea *	36	31	33	0	2	8
4 Method 2	ammonium-sulfate **	32	51	16	0	2	9
5 Method 2	partially reduced with dithiothreitol	29	24	23	24	4	11
6 Method 2	partially reduced and reoxidized	35	40	15	10	2	3

* 1 h incubation in 8 M urea at room temperature.

** Salting out with ammonium sulfate at 0.6 saturation, pH 7.0.

to Method 2 was treated with 8 M urea at room temperature, pH 7.5, for 1 h and then processed to determine the location of the labelled CM-peptides, carboxymethylated with the usual procedure (cf. Table II, line 3).

It must be emphasized that treatment with urea would not in any way irreversibly influence the enzymatic activity of amylase [11]. Moreover, even when the radioactivity is distributed over three different peptide spots, only 2 mol of SH groups per mol of amylase can be titrated. The peptides involved, peptide 2,3 and 4 have different amino acid composition and sequences; therefore no homology can be suspected.

It is of interest that not only urea treatment, but even salting out of the enzyme with ammonium sulfate (at 0.6 saturation, pH 7.0) produces a similar, though smaller effect, giving rise to more than two radioactive spots in the test for SH groups (Table II, line 4).

It is our interpretation, that treatment of the enzyme with concentrated urea leads to an intramolecular SH-disulfide exchange, which results in a micro-heterogeneous population of amylase molecules, having 3 different combinations of 2 SH groups each. This could happen if there is a disulfide bond in the vicinity of the SH groups present in the native amylase.

To examine this possibility, we have reduced amylase in presence of 3 mM EDTA and 20 mM dithiothreitol for 2 h at 0° C. Removing dithiothreitol by gel filtration, we found after this treatment 4 mol of SH groups per mol of amylase, confirming that one of the disulfide bonds is much easier to reduce than the rest [4,7]. Such a preparation was carboxymethylated with [^{14}C]bromoacetate in presence of 10 mM EDTA and then fully reduced and carboxymethylated with unlabelled bromoacetate. Four radioactive spots of equal in-

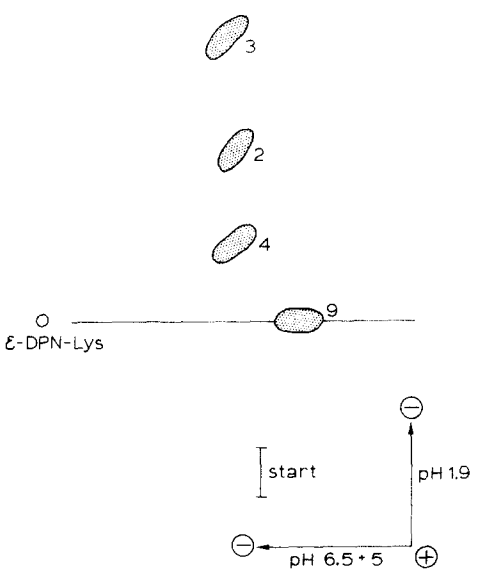


Fig. 4. Radioautogram of [^{14}C]CM-Cysteinyl peptides of tryptic hydrolysate of partially reduced amylase: 0.2 mM amylase solution was incubated with 20 mM dithiothreitol in 0.1 M Tris · HCl buffer (pH 8.5) containing 3 mM EDTA, at 0° C for 2 h. The mixture was then gelfiltered on a Sephadex G-50 column. For more details see Materials and Methods.

tensity were detected on the tryptic peptide map, corresponding to the neutral peptides 2,3,4 and the acidic peptide, peptide 9 (cf. Table II, line 5 and Fig. 4).

When a dithiothreitol-reduced amylase, having four SH groups, is left to stand in neutral solution at room temperature (the excess dithiothreitol having been removed by gelfiltration) after a few hours, two of the SH groups disappear. The resulting enzyme has been investigated and found to contain the remaining two SH groups in the peptides 2 and 4 mostly (cf. Table II, line 6).

As pancreatic amylase prepared by the method of Hatfaludi et al. [11] is known to be a mixture of two different forms, amylase I and amylase II, which can be separated by chromatography on DEAE-Sephadex [13], the microheterogeneity could be ascribed to this fact.

To examine this possibility, we prepared amylase by Method 1 and 2 and then separated amylase I and amylase II from both preparations. We found that the distribution of radioactivity over the peptide spots was identical in amylase I and II, in both amylase preparations. The differences were correlated only with the method of preparation. These results are therefore not shown separately, but are included in Table II (Methods 1 and 2, respectively).

Given the possibility of an intramolecular SH-SS exchange, which may lead to a mixture of molecules having somewhat different structures, we have inquired into the physiological significance of this phenomenon. We have investigated, whether the SS-SH rearranging enzyme of pancreas would catalyze a change in the localization of SH groups at physiological pH values and salt concentration. We have purified the SH-SS rearranging enzyme from pancreas according to Venetianer and Straub [20] and incubated different amylase preparations with it. The distribution of the SH groups (radioactivity of CM-peptides) was not changed by this treatment, although the rearranging enzyme was active in the test of regenerating ribonuclease from fully reduced ribonuclease [21]. Table II, line 1, also includes these results together with the controls.

Discussion

Our data led us to conclude that native pancreatic amylase has the two semiburied SH-groups located in the neutral tryptic peptides 2 and 4. These have been formerly identified in the carboxymethylated amylase by Fábián [8] as having the following aminoacid sequences:

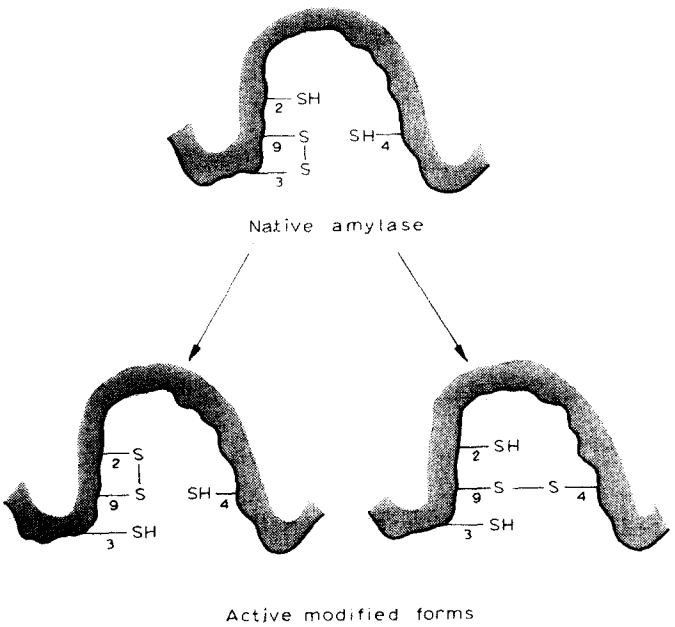
peptide 2: Leu.CM-Cys.Thr.Thr.Arg
peptide 4: CM-Cys.Asn.Asn.Val.Gly.Val.Arg

The disulfide residue, which is more easily reduced than the other three disulfide bridges, is formed between the cysteinyl residues of the peptides which are isolated in the carboxymethylated form as:

peptide 3 *: CM-Cys.Lys
peptide 9: Gly./CM-Cys_1,His_1,Tyr_x,Leu_{1-2},Ile_{1-2},Val_{0-1},Gly_1,Ala_1,Thr_1,Ser_1,Asx_2/.Lys

* Peptide 3 could be suspected to arise from peptide 9. However this possibility is excluded, since peptide 9 contains no radioactivity when peptide 3 is labelled (cf. Fig. 3).

These results may be explained by the schematic model shown in Scheme 1.



The evidence that the SH groups of peptides 2 and 4 are close to each other in the native enzyme, has been published earlier [4]. The present data indicate that in native amylase, the disulfide bridge between the cysteinyl residues of peptides 9 and 3 must be also nearby. Only in this case does one understand why relatively mild treatments, as with ammoniumsulfate or urea treatments, which do not impair enzyme activity, lead to an SH-disulfide exchange reaction within the amylase molecule.

Microheterogeneity has been detected in β-lactoglobulin [22], when either Cys-68 or Cys-70 is in the SH form. On the other hand, finding 2 SH groups per mol of paramyosin and isolating 3 different labelled peptides, have been used as proof of the heterogeneity of the paramyosin polypeptide chains [23]. It would be interesting to know whether these and similar microheterogenities are due to SH-SS interchange caused by handling of the proteins, as in the case of pancreatic amylase, or whether they really indicate in vivo heterogeneous polypeptide chains.

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