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A METHOD FOR DETECTING DISULFIDE INTERCHANGES
IN PROTEIN MODIFICATION STUDIES

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It is a privilege to be able to contribute a paper to a symposium in honor of Dr. Donald Van Slyke. The elegance and integrity of his experiments, his generosity in helping younger colleagues, his forthrightness and modesty in the face of many honors, and his dedication to science are an inspiration and standard of performance for all scientists and most particularly for those who are fortunate enough to be his colleagues. It is therefore an opportunity and a pleasure to be able to indicate our respect and admiration for Dr. Van Slyke by contributing to this symposium in his honor.

The work to be described here evolved from studies we were making on the correlation of protein structure and function. In order to determine the role of the amino acid residues in the action of enzymes, the most successful method developed so far is to modify residues by appropriate reagents and then correlate the modification with changes in the properties of the enzyme.¹ Various methods of modification have been developed in many laboratories but in all these studies it is of course assumed that the only modifications occurring are those measured. For this purpose careful analyses of all amino acids undergoing change are made. Fifteen years ago the inadequate methods of amino acid analyses made it essentially impossible

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to state that all modifications had been observed. In recent years, however, the elegant chromatographic methods of Stein and Moore² have made the determination of amino acids fairly straightforward. An evaluation of all covalent changes in amino acid residues resulting from a given treatment is therefore feasible and correlation of these changes with activity loss has been very rewarding in many studies. For such correlations to be meaningful it is of course clear that chemical changes must not be reversed or abolished during the course of the analysis. For example, it was recently observed that acid hydrolysis converted methionine sulfoxide back to the native methionine.³ Thus, any chemical procedure, such as an oxidation converting methionine to its sulfoxide, would go undetected if the subsequent conversion of protein to amino acids utilized an acid hydrolysis. This discovery has made it necessary to re-evaluate a fair amount of the older literature involving oxidation studies on proteins.

In the case of methionine oxidation the difficulty is merely methodological, and can be remedied by a different hydrolytic procedure. A fundamentally more difficult dilemma is faced in the case of potential S-S interchanges. If in the course, for example, of an oxidation study various amino acid residues are oxidized, the rate of oxidation of these residues can be correlated with activity loss in order to establish which residues are involved and which residues play no role in enzyme action.¹ The numbers and the types of residues which are oxidized can be determined by appropriate hydrolytic procedures and analysis on the analyzer. However, an S-S interchange reaction (Figure 1a) is fundamentally unobservable by analysis for amino acids, since the number of cystine residues is unchanged.

All molecular information distinguishing structure I from structure II (Figure 1a) is destroyed by total hydrolysis of any kind. Only the detailed separation of all the cystine peptides from the modified protein and comparison of these cystine peptides with those from the native enzyme would establish whether S-S interchange had occurred. Since such an interchange might easily occur and, in the absence of peptide analysts, lead to a false identification of the residues responsible for loss of activity, it seemed essential to develop a method for detecting whether interchanges were occurring under the conditions of the modification studies.

If the interchanges are intermolecular, the new protein would in all probability have a molecular weight different from that of the native species. Such interchanges could therefore be detected by ultracentrifuge studies. On the other hand, intramolecular disulfide interchanges might have very little effect on the sedimentation rate of the protein and would probably go undetected. Since it is extraordinarily difficult to do a peptide analysis of the type indicated above for each modification study, some simpler approach was sought. In this study we developed a method to determine what we shall term an "interchange potential." By incubating the protein in the presence of an excess of a low molecular weight symmetrical disulfide RSSR followed by measurement of the amount of RS bound to the protein, it seemed possible to estimate the amount of interchange catalyzed by the modification conditions. Structure I would then give rise to structure III or to some structure intermediate between I and III of Figure 1b. Although the S-S bonds of the protein might be either more or less labile than this type of mixed disulfide interaction, such an interchange would nevertheless give an indication of the likelihood of such a

reaction occurring at a rate comparable to the other changes taking place during the interval of treatment. The success of this approach depends on being able to measure the amount of covalently bound half-disulfide (RS). This was accomplished by oxidizing all disulfides to sulfonic acids (Figure 2) and measuring the amount of free sulfonic acid (RSO_2OH) produced. The disulfide reagent was therefore chosen with consideration of the ease with which its sulfonic acid derivative could be measured.

In the present work we chose to use cystamine as the disulfide. After removal of unbound cystamine, the modified protein is oxidized with performic acid according to the method of Hirs.⁴ This gives taurine in addition to the protein derivative shown in structure IV of Figure 2. By using relatively high concentrations of cystamine, an estimation of the amount of interchange occurring can be obtained from the amount of taurine derived from the treated protein.

MATERIALS

α -chymotrypsin, 3 times crystallized, was purchased from the Worthington Biochemicals Corp. Cystamine (2-2'-dithiobis(ethylamine)·2HCl), A grade, was purchased from the California Corporation for Biochemical Research. On electrophoresis in pyridine-acetate buffer at pH 6.5, two components were resolved, the slower one being present in minor amounts. Taurine was purchased from the Sigma Chemical Company. Formic acid was 98-100% pure and purchased from Matheson, Coleman and Bell. Hydrogen peroxide was reagent grade Superoxol from Merck and Company. Citrate buffer was the usual 0.2 M, pH 2.2 buffer used for amino acid analysis by the Stein and Moore procedure.

METHODS

(a) Incubation.--Chymotrypsin, 1.6 mM (8 mM in cystine), was incubated with cystamine, 80 mM, under a variety of conditions (Table 1).
Some of these conditions are_{used} frequently in determinations of amino acid residues involved in activity.

(b) Determination of extent of interchange.--Reagents were removed from the bulk of the protein by passing the solution (5 ml containing 200 mg protein) through a column of Sephadex G-25, 2 cm x 35 cm. The protein was then lyophilized.

A weighed aliquot of the dried protein was dissolved in formic acid and oxidized with performic acid as described by Hirs.⁴ Reagents were removed from the oxidized products by lyophilization.

A weighed amount (50-100 mg) of the oxidized products was suspended in 3-5 ml of citrate buffer, vigorously mixed, and centrifuged. The protein remains largely undissolved. A 2-4 ml aliquot of the clear supernatant was applied to a 100 cm column of Dowex-50 for analysis by the Moore and Stein procedure. Taurine was shown to emerge at 80 ml as a well-resolved peak.

(c) Separation of disulfides of low molecular weight.--Glutathione and cystamine were separated from the mixed disulfide by high voltage (40 volts/cm) electrophoresis on paper in pyridine-acetate buffer at pH 6.5 (Figure 3). The sample was applied 10 cm nearer to the anode side of the center line, since the cationic mobility of cystamine is very high. This permits full utilization of the mobility differences between the disulfides to effect complete separation. After electrophoresis, the paper was stained for disulfides either with the nitroprusside-cyanide reagent or with the Brand and Kassel modification of Folin's uric acid reagent.⁵

RESULTS AND DISCUSSION

The results are summarized in Table 1. Disulfide interchange is quantitatively expressed as the number of moles of taurine liberated by performic acid from one mole of the enzyme.

The results demonstrate that under the conditions used for peroxide treatment, X-radiation, and photooxidation there is (with chymotrypsin) virtually no disulfide interchange. Under iodination conditions there is substantial interchange. The presence of a catalytic amount of thiol at pH 9 yields slight interchange. The presence of urea (7.5 M) at pH 8.8 caused the greatest extent of interchange, as one might reasonably expect.⁶ It is to be noted that under these conditions there is 5% of the maximum possible amount of interchange. It thus appears that the disulfide bonds of chymotrypsin even when the molecule is unfolded, are resistant to interchange.

From an investigation on disulfide interchange reactions Ryle and Sanger⁷ concluded that there are two mechanisms leading to interchange, one operative in strongly acid solutions, the other in mildly acid, neutral or alkaline solutions. For the latter case, which is the one of interest to us, they proposed a mechanism (equations 1-3) involving initial attack by OH^- to give the half disulfide anion R_1S^- .

The reaction between two disulfides is thus favored by high pH and by catalytic amounts of thiol, and is essentially the same as thiol-disulfide interchange.⁸ For at least some low molecular weight disulfides the over-all

reaction

has an equilibrium constant close to 1.⁹ The increased amount of interchange observed in our experiments at high pH is thus in accord with the observations and mechanism of Ryle and Sanger.

In order to evaluate the low total interchange observed with chymotrypsin, the formation of a mixed disulfide from two disulfides of low molecular weight was briefly investigated. A mixture of glutathione and cystamine when irradiated at pH 3 gave rise to the mixed disulfide (cf. Figure 3 and Table 2). This component did not appear in the absence of radiation. Interchange reactions in a similar system have previously been observed by Cavallini et al.¹⁰ Peroxide at the same pH, and brief incubation at pH 7.2, also gave rise to the new component. In the latter case approximately equal amounts of the three disulfides were obtained. Since little interchange was observed with the protein under these sets of conditions, one is led to conclude that the disulfide bonds of chymotrypsin are relatively inert to interchange.

It is clear that the "interchange potential" method is able to give an indication of the potential for exchange in aqueous solutions under first four methods involve oxidizing conditions or the type of various conditions. Although the conditions, i.e. free radical formation, which might be assumed to lead to exchange, little exchange actually occurred. In strongly basic solution, a condition which has been shown by previous investigators to cause interchanges, and in the presence of the oxidant ICl, easily detectable amounts of interchange were observed. When the protein was unfolded so that access to interior S-S groups was increased, very appreciable

observed. Even in this case, however, the amount of interchange was noticeably less than that of the model systems involving low molecular weight S-S compounds such as glutathione and cystamine. It seems clear, therefore, that the interchange in the protein chymotrypsin, and very probably in most other proteins containing disulfide bonds, is orders of magnitude less than that observed in small molecules in aqueous solution. The protein rates at low pH's with mild reagents such as H_2O_2 or X-radiation are sufficiently low to be insignificant. At high pH's for long intervals, however, such interchanges might be significant factors and should be looked for carefully.

A particularly valuable feature of this method is the ease of detecting small amounts of interchange. The taurine appears at a point in the chromatogram which is relatively free of other amino acids. Moreover, it is liberated from the protein by treatment with performic acid without the necessity of degrading the entire protein by acid hydrolysis or some equivalent means. Therefore, it is a simple matter to add very large amounts of protein in those cases in which the amount of S-S interchange is presumed to be small. A procedure which would require complete hydrolysis of the protein would flood the amino acid analyzer and might make it impossible for the column of resin to effect a quantitative separation of taurine.

SUMMARY

A method has been developed for the detection of the potential of S-S interchanges occurring in protein modification studies. The method involves the incubation of the protein with cystamine under the reaction conditions to be investigated. After removal of excess cystamine, the

protein is treated with performic acid and the liberated taurine is determined quantitatively on the amino acid analyzer. The method has been applied to chymotrypsin under a variety of oxidizing conditions, pH ranges, and other conditions which might be expected to give S-S interchanges. The method appears to be of general applicability to proteins containing disulfide bonds.

REFERENCES

- 1) Koshland, D. E., Jr., Adv. Enzymol. 22, 45 (1960).
- 2) Moore, S., Spackman, D. H., and Stein, W. H., Anal. Chem. 30, 1185 (1958).
- 3) Ray, W. T., and Koshland, D. E., Jr., J. Biol. Chem. 237, 2493 (1962).
- 4) Hirs, C. H. W., J. Biol. Chem. 219, 611 (1956).
- 5) a) Toennies, G., and Kolb, J. J., Anal. Chem. 23, 823 (1951).
b) Kassell, B., and Brand, E., J. Biol. Chem. 125, 115 (1938).
- 6) Kauzmann, W., and Douglas, R. G., Jr., Arch. Biochem. Biophys. 65, 106 (1956).
- 7) Ryle, A. P., and Sanger, F., Biochem. J. 60, 535 (1955).
- 8) Pihl, A., and Eldjarn, L., 4th Intern. Congr. Biochem. 13, 43 (1960).
- 9) Kolthoff, I. M., Stricks, W., and Kapoor, R. C., J. Am. Chem. Soc. 77,
4733 (1955).
- 10) Cavallini, D., Mondovi, B., Giovanella, B., and De Marco, C., Science
131, 1441 (1960).

Table I

Interchange Potential of Chymotrypsin Under Various Conditions

Reagent	Conditions			Interchange (Mole Tau/Mole ChT)
	pH	Time (Hours)	Temp. (°C)	
H ₂ O ₂ , 20 mM	3.0	18.	23.5	0.006
X - radiation, 1.25×10^5 r	3.0	2.0	25.	.007
Visible Radiation + Methylene Blue	7.0	0.0833	25.	.012
Cysteamine, 0.26 mM	9.0	3.58	24.	.05
ICl, 26. mM	6.8 + 8.9	0.58	0.	.13
Urea, 7.5 M	8.8	24.	25.	.52

Chymotrypsin was present at a concentration of 1.6 mM and cystamine at a concentration of 80 mM. The figures have been corrected for the control interchange value of 0.003.

Table II

Disulfide Interchange Between Glutathione (GSSG) and Cystamine

Solution	Treatment	Electrophoretic Component		
		A	B	C
Cystamine	None	++	0	0
Glutathione (GSSG)	None	0	0	++
Cystamine + GSSG	None	++	0	++
" "	57×10^3 r, pH 3	++	+	++
" "	H ₂ O ₂ , pH 3	++	+	++
" "	pH 7.2, 6 mins.	++	++	++
Electrophoretic "Mobility" (cm ² hr ⁻¹ volt ⁻¹)		-0.9	-0.1	+0.25

A, B, and C refer to positions on the paper strip, as described in Fig. 3.

Fig. 1a. Disulfide Interchange in a Protein

Fig 1b. Disulfide Interchange Between a
Protein and a Symmetrical Disulfide
Present in Excess

Fig. 2. Measurement of the Disulfide Interchange Potential

Fig. 3. Separation of Disulfides by Paper Electrophoresis

Electrophoresis was carried out in Pyridine - Acetate Buffer, pH 6.5, in a field of 38 volts per cm. for 45 minutes. The strips were stained either with the nitroprusside-cyanide reagent or with the Brand and Kassell modification of the uric acid reagent. A is cystamine, C oxidized glutathione, and B the mixed disulfide.