REACTIVITY OF CARBOXYL GROUPS IN MODIFIED PROTEINS

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The reactivities of carboxyl groups to carbodiimides in non-reduced, *S*-carboxymethyl, and *S*-cyanoethyl proteins have been compared. When disulphide bonds are split, a decrease in reactivity was found, relatively minor in the *S*-carboxymethyl derivative and almost complete loss of reactivities in the *S*-cyanoethyl derivative.

1. Introduction

Reagents capable of reacting with carboxyl groups of proteins have, until recently been unsatisfactory.

Koshland's development of the use of water soluble carbodiimides in the presence of a nucleophile [1] for this purpose has shown great promise and has been used both for the determination of total carboxyl content [1, 2] and to estimate their reactivities in native proteins – particularly enzymes [3]. The presence of a nucleophile in the reaction is essential, as otherwise the activated carboxyl group may participate in cross-linking reactions with protein nucleophilic groups [1]. For the determination of total carboxyl content, Koshland used water soluble carbodiimides in the presence of strong denaturing reagents (5 M guanidine HCl and 8 M urea) and a nucleophile, e.g. glycine methyl ester [1]. The extent of reaction was then determined by subsequent hydrolysis and analysis for additional glycine residues. The proteins studied by Koshland in this way all contained intact disulphide bonds, and since the intention of the present work was to study the reactivity of carboxyl groups in a reduced and alkylated low-sulphur protein from wool, it was of interest to see that the method had been applied to some *S*-carboxymethylated proteins [4]. However in applying the method to *S*-cyanoethyl wool protein, no reaction occurred and it was decided to test for completeness of reaction in several derivatives. An anomalous behaviour was also found for reduced and alkylated bovine serum albumin, although the intact protein behaved normally. In addition to analysis for incorporated groups, the extent of reaction was also followed by carboxyl group titration.

2. Materials

Crystalline bovine serum albumin (Cohn fraction V) was obtained from Commonwealth Serum Laboratories, Melbourne, Australia. The *S*-carboxymethyl derivative was prepared by reduction with 2-mercaptoethanol in the presence of 8 M urea followed by alkylation with iodoacetic acid [5] and the *S*-cyanoethyl derivative by the method used for wool proteins [7]. The low-sulphur protein from wool, component 8 was prepared by the method of Thompson and O'Donnell [6] in the *S*-carboxymethyl form, or by the method of Frater [7] as the *S*-cyanoethyl derivative. The water soluble carbodiimide, *N*-(3-dimethyl amino propyl)*N*-ethyl carbodiimide HCl (EDC) was obtained from Fluka. Glycine methyl ester HCl and L-arginine methyl ester di-HCl were prepared by the reaction of the amino acid with methanol in the presence of thionyl chloride [8]. The melting points were 175° and 194° respectively. Glycinamide (m.p. 186–190°) was prepared by the method of Bergell [9] and L-argininamide HCl was purchased from Miles laboratories. Guanidine hydrochloride was prepared from the carbonate by the method of Tanford [10]. It was then crystallized once from dry methanol. All other reagents used were of AR quality and were used without further purification, except that solutions of urea were

deionized by passage through a mixed-bed ion exchange resin.

3. Methods

For carboxyl group modification the conditions of Koshland [1] were followed, with the exception that 6 M guanidine HCl was used. The reaction was allowed to proceed for 3 hr, and then stopped by the addition of acetic acid. After extensive dialysis against water, the protein was freeze dried and samples taken for hydrolysis and amino acid analysis [11]. To estimate unreacted carboxyl groups, a sample of the protein (30–50 mg) was dissolved in 5 ml 6 M guanidine hydrochloride, the pH adjusted to 9, and then titrated with 0.1 N HCl added from a micro syringe. The pH after each addition was measured on a Radiometer pH stat. Blank titrations were carried out on 5 ml 6 M guanidine HCl, and the titration curves corrected for this.

Carboxyl group titrations on the proteins (SCM component 8, SCM albumin, and non-reduced albumin) before reaction with the carbodiimide gave values agreeing within 5% of the total free carboxyl content as determined by amino acid analysis. The values were corrected for amide content, determined by the micro-Kjeldahl method.

4. Results

4.1. *Reaction with unmodified albumin*

50 mg of non-reduced BSA was treated with EDC in the presence either of L-argininamide or glycine methyl ester as the nucleophile. After dialysis and freeze drying, a sample of the protein was titrated with acid, and there was no detectable acid uptake between the pH values of 5 and 2, indicating that all of the carboxyl groups had reacted. Amino acid analysis of the sample showed the addition of 112 moles of arginine per mole of BSA. Assuming an amide content of 27.3% of the total Asp + Glu content [12], the number of free carboxyl groups was 105 moles. The slight excess of arginine incorporated over the carboxyl content may be due to reaction with tyrosine residues [13]. Similar results were obtained using glycine methyl ester as a nucleophile.

4.2. *Reaction with S-carboxymethyl albumin and S-carboxymethyl component 8*

Table 1 shows the values obtained for carboxyl group content and number of moles of arginine incorporated in the two proteins when either arginine methyl ester or argininamide was used as the nucleophile. In each case, only about 80% of the carboxyl groups had reacted. Different conditions were tried to get complete reaction, for example, longer periods of reaction (up to 24 hr), different denaturing agents (5 M LiBr, 8 M urea, and 5 M LiBr in 50% DMSO*), and higher temperatures (50° overnight). In all cases there was still about 20% of the carboxyl groups unreacted.

4.3. *Reaction with S-cyanoethyl-albumin and S-cyanoethyl-component 8*

With both these derivatives, carboxyl group titration and amino acid analysis indicated that no reaction had occurred when the normal reaction conditions were used. The 4 nucleophiles used, arginine methyl ester, argininamide, glycine methyl ester and glycinamide each gave the same result. Again, different reaction conditions were tried without success except that about 20% of the carboxyl groups in SCE-component 8 could be substituted when the reaction was carried out in 5 M LiBr containing 50% DMSO (60°, overnight), but extensive loss of *S*-cyanoethyl cysteine residues occurred, and no real conclusions could be drawn.

Table 1
Carboxyl group content and moles of arginine incorporated before and after reaction with carbodiimide and L-argininamide HCl.

	Total no. free COOH available per mole	No. COOH groups found by titration	Moles arginine extra found per mole
SCM albumin	162	25	140
SCM component 8	83	15	70

* *Abbreviation*:
DMSO: dimethylsulphoxide.

5. Discussion

A comparison of the reactivities of the carboxyl groups of various derivatives of serum albumin, showed that reaction is complete in the non-reduced form, and the extent of reaction decreases when the disulphide bonds are broken. The *S*-carboxymethyl form shows a slight decrease in reactivity, whereas the *S*-cyanoethyl derivative is completely unreactive. The same effect is seen for the wool protein, although it was not possible to investigate the non-reduced form as component 8 is a protein extracted from wool by breaking disulphide bonds. No conditions have been found so far for exposing these unreactive carboxyl groups and their lack of reaction cannot be explained by formation of covalent bonds, as they are still fully accessible to protons.

The decrease in reactivity cannot be explained by any alteration in the activity of the carbodiimide in the presence of substituted sulphur groups. This was shown by allowing the reaction with *S*-cyanoethyl albumin to proceed for 3 hr as described above, adding 50 mg of non-reduced albumin and reacting for a further 3 hr. Carboxyl group titration of the product showed no increase over that found for *S*-cyanoethyl albumin alone, demonstrating that the carbodiimide was still reactive in the presence of *S*-cyanoethyl protein.

A likely conclusion is therefore that when the protein chains are freed of configurational restraints imposed by the presence of disulphide bonds, they are free to assume positions of minimum free energy. Thus extremely strong interactions between carboxyl groups and amino (or guanidino) groups may occur. That these bonds may not be broken in the presence of 6 M guanidine HCl is perhaps not surprising in view of the conclusion by Nozaki and Tanford [14] that this reagent is not particularly effective in breaking salt linkages. The effect is less marked with the *S*-carboxymethyl derivatives, and this may be due to stronger repulsive forces operating in a highly charged molecule [15]. However, the *S*-cyanoethyl derivative carries no extra charge and the repulsive forces would be minimal. The dramatic difference between the two forms of serum albumin carrying the same charge (non-reduced and *S*-cyanoethyl), gives support to the above conclusion. The solubility difficulties and tendency to irreversible aggregation shown by reduced and alkylated disulphide containing proteins [16] may be due to this effect.

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