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A case of spurious product formation during attempted resynthesis of proteins by reverse proteolysis

Some batches of 'pure' glycerol contain cross-linking agents

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In cases where enzyme-catalysed synthesis of a peptide bond is being used to re-form a protein from two large peptide fragments, the organic co-solvent chosen has so far been glycerol, for most solvents in use in small-molecule systems are potent protein denaturants. We have found, however, that impurities contaminating certain batches of glycerol are effective in cross-linking the complexes formed by these peptide fragments, thus mimicking the enzyme-catalysed process. In one such case, the reported re-formation of cytochrome *c* from a two-fragment complex system, cytochrome *c*-T, the extent and rate of conjugate formation duplicates that reported for enzymic resynthesis. We observed no difference between mixtures containing or lacking enzyme. We warn of the danger of confusion possible to those engaged in studies of resynthesis, and suggest a simple control of purchased glycerol to avoid it. We recommend similar caution to those (X-ray crystallographers and others) who seek to stabilize protein solutions by adding large quantities of glycerol.

Protein semisynthesis is proving to be a very useful technique for the generation of analogues of academic, clinical and commercial interest (Offord, 1980, 1983). The condensation of large peptide fragments is one of the most technically demanding aspects of successful protein semisynthesis. As these fragments are often generated by limited enzymic cleavage techniques, then, in principle, reversal of the cleavage reaction would be a highly convenient method of performing such condensations.

Recent studies have demonstrated the feasibility of reversing proteolysis (for a review see Chaiken *et al.*, 1982) and have demonstrated the resynthesis of active proteins from large fragments thereof. Indeed, such resyntheses exhibit the desirable characteristics expected of them when contrasted with chemical methods of coupling.

In practice there are two barriers to enzymic resyntheses from large fragments. The thermodynamic barrier may be overcome by inclusion of an organic solvent in the system. The equilibrium in most proteinase reactions is very much in favour of cleavage, but the equilibrium constant can be shifted towards synthesis by inclusion of organic solvent. The organic co-solvent both lowers the concentration of one of the reagents, water, and raises the pK of the α-carboxy group and thus the concentration of the protonated form of the reacting peptide. The organic solvent in these systems is always glycerol, in contrast with other enzymic resynthesis systems, as this compound does not appear to act as a protein denaturant.

The kinetic barrier to resynthesis, posed by the limited solubility of large fragments relative to small-molecule systems in semi-aqueous solution, can be surmounted if the two fragments associate to give a structure resembling that of the product, for in such a case the effective local concentration of the two reacting groups will be considerably enhanced.

Hence all the reported examples of enzymic resynthesis from large fragments involve products of limited cleavages that form such non-

Abbreviations used: Msc, methanesulphonylethyloxycarbonyl; ONSu, succinimido-oxy; Dmp, 2,2′-dimethylpyrimidylornithine; Mes, 4-morpholine-ethanesulphonate; SDS, sodium dodecyl sulphate.

covalent complexes, namely ribonuclease S (Richards & Wykoff, 1971), staphylococcal nuclease-T (Chaiken & Anfinsen, 1971), cytochrome *c*-T (Harris & Offord, 1977), human somatotropin (Graf *et al.*, 1981), or, as in the case of soya-bean trypsin inhibitor, where the fragments are held together by disulphide bridges (Sealock & Laskowski, 1969). All five cases have been reported as susceptible to enzymic resyntheses under the conditions outlined above (Homandberg *et al.*, 1978; Homandberg & Laskowski, 1979; Komoriya *et al.*, 1980; Graf & Li, 1981; Juillerat & Homandberg, 1981).

While examining the potential of the reported resynthesis of cytochrome *c* from cytochrome *c*-T as a tool for semisynthesis, we noticed apparent conjugate formation even in the absence of enzyme. The phenomenon was also seen to occur in the case of ribonuclease S. Characterization of the products after isolation under conditions that would cause dissociation of the starting non-covalent complexes shows them to resemble the parent proteins in molecular mass, biological activity and amino acid composition. The yield of conjugate varies with the source of the glycerol employed, and, in the case of cytochrome *c*-T, the yield can under some conditions equal that reported for enzymic synthesis with clostripain. We were not able to increase the rate of conjugate formation by adding clostripain.

We speculated that the conditions employed might have led to a degree of activation of the *C*-terminal carboxy group comparable in magnitude with that produced by lactonization of the *C*-terminal homoserine residue of CNBr-cleavage fragments. We therefore checked the possibility that conjugate formation might result from the spontaneous re-formation of the peptide bond between the two fragments (Proudfoot & Wallace, 1983). [The presence of *C*-terminal homoserine lactone adjacent to the *N*-terminus of the other fragment leads to spontaneous resynthesis of cytochrome *c* and of pancreatic trypsin inhibitor from non-covalent complexes of their CNBr-cleavage fragments (Corradin & Harbury, 1974; Dykes *et al.*, 1974).] However, the peptide bond that would result from spontaneous resynthesis of cytochrome *c* from cytochrome *c*-T was not found.

We analysed the different batches of glycerol employed in these studies and showed traces of aldehydes to be present in various amounts. The batch of glycerol giving rise to the greatest amount of conjugate was shown to contain an identifiable amount of acrolein. Addition of acrolein to mixtures made up with glycerol not demonstrating this property led to a high yield of product resembling the conjugate produced in the original experiments.

Experimental

Materials

Horse heart cytochrome *c* (type III), bovine pancreatic ribonuclease A (type XII-A), bovine pancreatic ribonuclease S-peptide (grade XII-PE), bovine pancreatic ribonuclease S-protein (grade XII-PR) and 2',3'-cyclic CMP were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) Clostripain was obtained from Boehringer Mannheim (Mannheim, W. Germany) and from Sigma Chemical Co. Endoproteinase Arg-C was from Boehringer Mannheim. Thrombin (90 N.I.H. units/mg) was obtained from Hoffmann–La Roche (Basel, Switzerland). Trypsin [1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK')-treated] and chymotrypsin were obtained from Millipore Corp. (Freehold, NJ, U.S.A.). Methanesulphonylethyl succinimidocarbonate (Msc-ONSu) was kindly given by Dr. G. I. Tesser (Catholic University, Toernooiveld, Nijmegen, The Netherlands. Methyl acetimidate hydrochloride was prepared by the method of Hunter & Ludwig (1962). Acrolein was Merck (Darmstadt, W. Germany) 'electron microscope' grade. Glycerol was analytical grade from Fluka (Buchs, Switzerland) or Merck, or Extra Pure grade from Merck. All other reagents used in this work were analytical grade from Merck and Fluka.

Preparation of cytochrome c fragments

Fragments 1–38 and 39–104 of cytochrome *c* were prepared by tryptic digestion of the fully lysine-protected protein. 19-*N*-ε-Acetimidyl-cytochrome *c* was prepared by the method of Wallace & Harris (1984) and cleaved by the method of Harris & Offord (1977). The fragments were not deprotected, as this protecting group causes retention of the positive charge and biological properties of the protein. 19-*N*-ε-Msc-cytochrome *c* was prepared and cleaved, and the fragments were deprotected, by the method of Westerhuis *et al.* (1979). 19-*N*-ε-Citraconyl-cytochrome *c* was prepared and cleaved, and the fragments were deprotected, by the method of Juillerat *et al.* (1980).

The fragments were purified by ion-exchange chromatography, with the system described by Wallace & Harris (1984). Their homogeneity was checked by SDS/polyacrylamide-gel electrophoresis and their integrity by their ability to form a complex exhibiting an absorption band at 695nm (Harris & Offord, 1977).

SDS/polyacrylamide-gel electrophoresis

Gradient slab gel electrophoresis (gradient 15–25% in acrylamide) in the presence of SDS was by the method of Laemmli (1970).

To follow cytochrome *c* synthesis, 25 μl portions (50 μg of complex) of the synthesis mixtures were diluted with 25 μl of the denaturing buffer and heated at 100°C for 5 min. For purity checks of the fragments, 60 μg of each fragment was similarly treated. Ribonuclease A synthesis was monitored by diluting 10 μl of the resynthesis mixture with 10 μl of denaturing buffer, heated at 100°C for 5 min and then for a further 1 h at 60°C to eliminate the effect of glycerol stabilization observed by Bello (1969).

The samples were subjected to electrophoresis at 25°C with a constant current of 25 mA for 5–6 h. The gels were stained overnight in 0.25% Coomassie Blue R-250, dissolved in methanol/water/acetic acid (9:9:2, by vol.) and destained in the same solvent.

The stained slabs were cut into strips and scanned at 546 nm with an Isco gel scanner.

Attempted resynthesis of cytochrome c from fragments

The conditions for enzymic resynthesis described by Juillerat & Homandberg (1981) were employed. Four different lots of the Boehringer enzyme were used, with specific activities ranging from 38.5 to 147 units/mg, and one lot of enzyme from Sigma with a specific activity of 71 units/mg. Clostripain activity after incubation in the resynthesis mixture was assayed as described in *Biochemica Information II*, p. 52 (Boehringer, Mannheim). Other enzymes employed were endoproteinase Arg-C and thrombin, both dissolved in 30 mM-NH_4HCO_3, pH 7.8, with unprotected fragments, and trypsin (in 1 mM-HCl) for acetimidylated fragments. Each experiment including enzyme in the reaction medium was accompanied by a control that lacked enzyme.

A series of control mixtures (lacking enzyme) were prepared with glycerol from different sources. To some of these, acrolein (1:500 and 1:1000, v/v) was added. After 3 days' incubation, the amount of product formed was observed by SDS/polyacrylamide-gel electrophoresis.

Isolation and characterization of the cytochrome c product

The product was separated from the resynthesis mixture after an incubation period of 7–10 days by gel filtration on a column (150 cm × 2.6 cm) of Sephadex G-50 (fine grade) in 7% (v/v) formic acid.

Biological activity was determined with an oxygen electrode in the depleted-mitochondria system of Jacobs & Sanadi (1960) as described by Harris & Offord (1977).

We prepared a one-dimensional chymotryptic peptide map, using parallel digestions of 18 nmol of horse cytochrome *c* and the product of attempted resynthesis. The materials were denatured with acetone, and redissolved in 0.5% NH_4HCO_3, at a concentration of 10 mg/ml. Portions (4 μl) of the chymotrypsin solution (1 mg/ml) were added every 10 h, and digestion was performed at room temperature (Margoliash & Smith, 1962). After 30 h, the digestion was stopped by freezing. Then 10 μl portions of the digests were subjected to paper electrophoresis at pH 6.5 (3000 V for 30 min) and the electrophoretograms were developed with ninhydrin/cadmium and Sakaguchi reagents.

G.l.c./mass-spectral analysis of horse cytochrome *c* and of the product were as previously described (Rose *et al.*, 1983).

Resynthesis of ribonuclease from fragment mixtures

Ribonuclease S-protein and ribonuclease S-peptide were dissolved in 100 mM-Mes buffer, pH 6.2, containing 100 mM-$CaCl_2$, and 9 vol. of glycerol was added to give a final concentration of the complex, ribonuclease S, of 5 mg/ml. The formation of covalently conjugated ribonuclease was followed by SDS/polyacrylamide-gel electrophoresis and the development of biological activity. A series of mixtures was prepared with glycerol from different sources. Acrolein (1:500, v/v) was added to a parallel set of these mixtures and incubated for 3 days.

Enzymic assay of ribonuclease A

Ribonuclease activity was assayed by the method of Crook *et al.* (1960) with 2',3'-cyclic CMP as substrate in 50 mM-Tris/HCl buffer, pH 7. The enzyme was added directly as a 10-fold dilution of the glycerol-containing resynthesis mixture. To differentiate activity of ribonuclease A from that of the active complex, ribonuclease S, the substrate solution was adjusted to 40% (v/v) dioxan, conditions under which ribonuclease S is dissociated and inactivated (Homandberg & Laskowski, 1979).

Analysis of glycerol stocks

Aldehyde content in the four different stocks of glycerol was analysed with Schiff reagent as described by Vogel (1956). Acrolein was identified by high-performance thin-layer chromatography on silica gel G, after formation of the dinitrophenylhydrazone derivatives, with chloroform/hexane/ethyl acetate (10:2:1, by vol.) as solvent, and comparison of the R_F values of the derivatives with those of standards.

Results and discussion

The purified fragments all electrophoresed as single bands on SDS/polyacrylamide-gel electrophoresis. No evidence of residual cytochrome *c*

was seen, even at high loadings. Fragments 1–38 and 39–104 and acetimidyl fragments could be combined to make productive complexes, cytochrome *c*-T and acetimidyl-cytochrome *c*-T, with the spectrophotometric and biological properties reported by Harris & Offord (1977) and Westerhuis *et al.* (1979). These complexes when electrophoresed on SDS/polyacrylamide gels were completely dissociated, giving two bands with mobilities corresponding to those of fragments 1–38 and 39–104.

Gel electrophoresis of samples of resynthesis mixtures showed the appearance of a haem-containing band co-migrating with a cytochrome *c* marker. The intensity of this band as measured by densitometric scanning of stained gels increased with time to a maximum reached after about 7–10 days. The band was also present in the control mixture lacking enzyme. Solutions of complex kept in 0.1 M-NH_4HCO_3 did not show any production of this band. Product formation thus seems to be dependent on exposure to the glycerol-containing buffer.

In the comparative trials it was seen that the intensity of the product band varied with the source of the glycerol. On incubating four samples of both cytochrome *c* and ribonuclease resynthesis mixtures in glycerol from different lots, the amount of conjugate formed was significantly greater for both proteins with one particular lot of glycerol (Fig. 1). The mixtures of cytochrome *c* fragments were filtered on Sephadex G-50 in 7% formic acid and the yields of conjugate varied from 1 to 20%. Mixtures yielding the most conjugate also seemed capable of producing significant quantities of homopolymers of the fragments. When acrolein was added to a batch of glycerol that did not give rise to significant conjugate formation, a high yield of cross-linked product and some general polymerization of fragments were observed (Fig. 2).

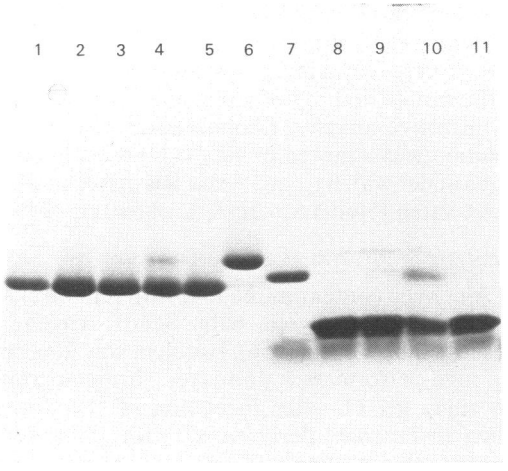

Fig. 1. *SDS/polyacrylamide-gradient-slab-gel electrophoresis, after 5 days' incubation of ribonuclease S and cytochrome c-T in glycerol from different sources (for conditions see the text)*

Lane 1, ribonuclease S in water; lanes 2–5, ribonuclease S in glycerol samples 1, 2, 3 and 4; lane 6, ribonuclease A marker; lane 7, cytochrome *c* and fragment 1–38 markers; lanes 8–11, cytochrome *c*-T in glycerol samples 1, 2, 3 and 4.

Mixtures prepared with acetimidylated fragments of cytochrome *c* were observed to give the same qualitative results in the comparative trials, and to show an increase in conjugate formation on the addition of acrolein. However, yields of conjugate appeared to be considerably lower than those obtained with the unprotected fragments.

The inclusion of clostripain, or two other arginine-specific enzymes, did not increase yields of conjugate, yet assays of enzyme activity showed that the reaction conditions were not inactivating the enzyme. After 14 days' incubation in a reaction medium producing a 20% yield of conjugate, clostripain had 60% of its original activity. The control mixture kept under sterile conditions also yielded the cytochrome *c*-like material, and hence extraneous enzyme was not responsible for its production.

Should the conjugate observed have been the result of re-formation of the cleaved peptide bond between residues 38 and 39, then chymotryptic digests of the product molecule from cytochrome *c* fragment mixtures would have contained the decapeptide 37–46 found in digests of native cytochrome *c*. This peptide is easily identifiable, as it shows a characteristic yellow colour reaction with ninhydrin/cadmium, glycine being *N*-terminal (Margoliash & Smith, 1962). The mobility of this peptide relative to aspartic acid = 1 was calculated to be 0.51 by the method of Offord (1966). A yellow peptide with this mobility was observed in the peptide map of horse cytochrome *c*, but not in that of the putative product. Digests of the conjugate did, however, show a yellow peptide with a mobility (0.68) corresponding to that calculated for the dipeptide Gly-Arg (0.68), suggesting that the *C*-terminal of fragment 1–38 in the conjugate is still free.

This result was confirmed by g.l.c.–mass-spectral analysis. Native cytochrome *c*, after condensation with pentane-2,4-dione to convert arginine residues into residues of 2,2'-dimethylpyrimidylornithine (Dmp), digestion with a mixture of trypsin and chymotrypsin, acetylation and permethylation, gives rise, among other peptides, to the peptide Gly-Dmp-Lys, which may be identified by g.l.c.–mass-spectral analysis (Rose *et al.*, 1983). Rose *et al.* (1983) describe the identification

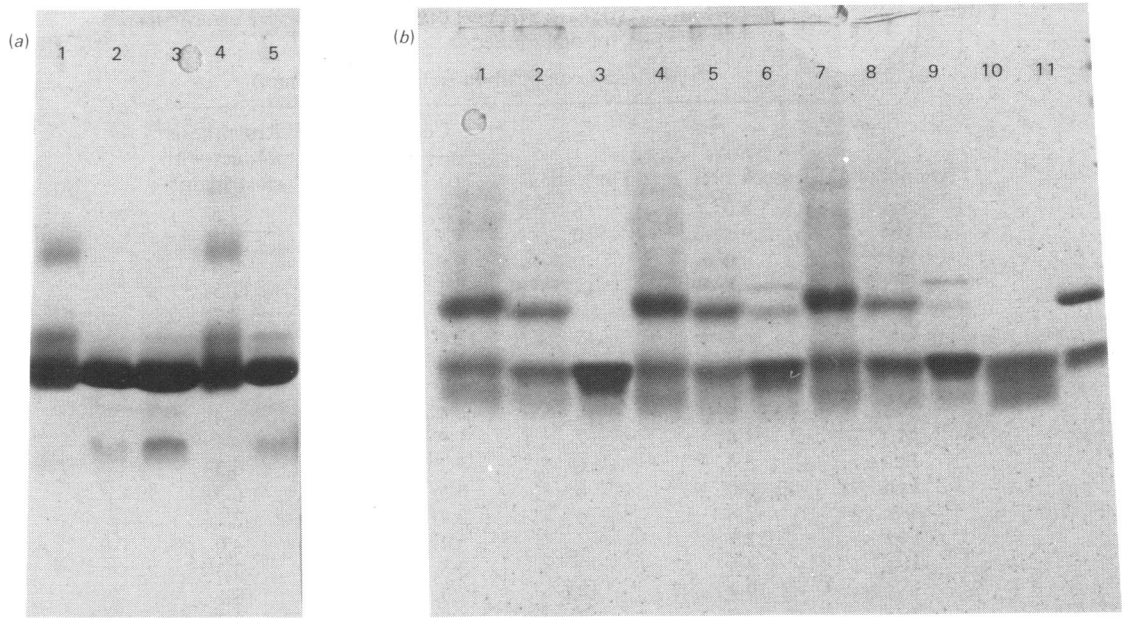

Fig. 2. *SDS/polyacrylamide-gradient-slab-gel electrophoresis, showing the effect of addition of acrolein to ribonuclease S and cytochrome c-T solutions after 3 days' incubation (for conditions see the text)*

(*a*) Ribonuclease S. Lane 1, in glycerol 4 with acrolein (1:500); lane 2, in glycerol 4; lane 3, in H_2O; lane 4, in glycerol 3 with acrolein (1:500); lane 5, in glycerol 3. (*b*) Cytochrome *c*-T. Lane 1, in glycerol 4 with acrolein (1:500); lane 2, in glycerol 4 with acrolein (1:1000); lane 3, in glycerol 4; lane 4, in glycerol 3 with acrolein (1:500); lane 5, in glycerol 3 with acrolein (1:1000); lane 6, in glycerol 3; lane 7, in glycerol 2 with acrolein (1:500); lane 8, in glycerol 2 with acrolein (1:1000); lane 9, in glycerol 2; lane 10, in H_2O (lane 11, cytochrome *c* and fragment 39–104 markers).

of the dipeptide Gly-Dmp, but not of Gly-Dmp-Lys, when a mixture of fragments 1–38 and 39–104 is treated similarly. When a sample of the conjugate isolated by gel filtration in 7% formic acid was analysed in the same way, Gly-Dmp was identified by g.l.c.–mass-spectral analysis in good yield and there was no evidence of any Gly-Dmp-Lys.

Amino acid analyses of the conjugate (Table 1) were similar to those of native cytochrome *c* and the product of the clostripain-containing mixtures described by Juillerat & Homandberg (1981). The low lysine content observed in these products may be significant.

When conjugate from mixtures of cytochrome *c* fragments was tested in the depleted-mitochondria assay it showed a specific activity of about 10% compared with native cytochrome *c*. The non-covalent complex, cytochrome *c*-T, has 30% of the biological activity shown by native cytochrome *c* in this assay (Wallace, 1984).

When ribonuclease S was incubated under the conditions that lead to cytochrome *c* resynthesis, we observed the development with time of a band that migrated at the position of the ribonuclease A marker on SDS/polyacrylamide-gel electrophoresis. Prolonged incubation of samples of the reaction mixture in the denaturing electrophoresis buffer at 60°C did not cause this band to disappear. Enzymic activity of the crude mixture under conditions that dissociate and inactivate ribonuclease S (40% dioxan) showed a parallel development of 2′,3′-cyclic CMP-hydrolytic activity.

We have clearly demonstrated that the conditions typically employed in enzymic resynthesis reactions can generate conjugates that mimic the genuine product of such resynthesis. The considerably lower yields of conjugate when lysine residues are acetimidylated, and the low lysine contents observed on amino acid analysis of conjugates, suggest that lysine residues may contribute to the cross-links in such conjugates. The agent responsible, which occurs in commercial preparations of glycerol, appears to be acrolein. The proportion present in these commercial samples is very variable, so that conjugate yield varies from negligible to heavy. There is thus a very real danger of this side reaction occurring to a significant

Table 1. *Amino acid compositions of resynthesis products compared with native cytochrome c*
–, Not determined.

	Amino acid composition (mol of residue/mol)				
			Conjugate		Resynthesis product with clostripain*
Amino acid	Native cytochrome *c*	Theoretical	(i)	(ii)	
Asx	8.0	8	9.2	8.8	7.5
Thr	9.5	10	7.7	7.8	9.1
Ser	0.0	0	0.0	0.0	0.0
Glx	12.4	12	15.1	13.6	11.5
Pro	4.4	4	–	–	3.7
Gly	12.4	12	12.2	10.8	12.4
Ala	6.1	6	6.0	6.0	5.5
Cys	–	2	–	–	–
Val	2.8	4	4.4	3.1	3.4
Met	1.6	2	1.7	1.9	–
Ile	5.0	6	5.2	6.4	6.3
Leu	6.0	6	6.5	6.8	6.3
Tyr	2.8	4	3.4	4.1	3.1
Phe	3.5	4	3.4	3.8	4.0
His	3.5	3	2.8	2.5	3.3
Lys	19.4	19	17.0	15.0	18.1
Arg	2.0	2	1.7	2.0	2.3

* Juillerat & Homandberg (1981).

extent in enzymic resynthesis studies and deceiving the investigator. We suggest that any batch of glycerol intended for use in such studies be evaluated prior to use with the commercially available complex, ribonuclease S, and that controls lacking the proteinase accompany all resynthesis mixtures.

The rapidity of the attainment of high yield, and the quality of the product, when compared with acrolein-induced conjugate formation, and the rigorousness of the product characterization in the published resynthesis of ribonuclease (Homandberg & Laskowski, 1979), exclude the possibility that anything other than genuine enzymic resynthesis was responsible for product formation.

However, some doubt must remain in the case of the reported resynthesis of cytochrome *c* from cytochrome *c*-T (Juillerat & Homandberg, 1981). All the characteristics of this resynthesis can be duplicated by the presence of acrolein at concentrations observed in commercial samples of glycerol of high purity. Although the product was reported to be reducible by lactate dehydrogenase, the rate of reduction was not quantified. Quantification of biological assays appears, in fact, to be the simplest way to distinguish between conjugate and the genuine resynthesis product, native cytochrome *c*. Furthermore, there are anomalies in the cytochrome *c* system that do not obtain in the other reported cases of enzymic resynthesis. (i) The enzyme used for resynthesis, clostripain, is not that employed for cleavage. Under physiological conditions clostripain will not catalyse the cleavage that it is supposed to reverse. (ii) The cleavage of cytochrome *c* by trypsin will only occur when the molecule is denatured either by the addition of a charge-changing amino-protecting group, in the citraconyl or Msc cases, or by acetone pretreatment, in the acetimidyl case. These two observations suggest that cleavage of the 38–39 bond in the native [or near native, as in acetimidyl-cytochrome *c* (Wallace, 1984)] structure is very difficult or impossible to achieve, probably for steric reasons. Reversal of such a cleavage in the near-native cytochrome *c*-T structure to re-form the native molecule would be expected, for the same reasons, to be equally difficult.

The danger of artifact formation is not restricted to studies on semisynthesis. Many workers (X-ray crystallographers and others) stabilize protein solutions against denaturation by adding large amounts of glycerol. Should it happen that the glycerol in question is contaminated by acrolein, it seems quite possible that the protein, rather than being protected, would be damaged.

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References

Bello, J. (1969) *Biochemistry* **8**, 4535–4541

Chaiken, I. M. & Anfinsen, C. B. (1971) *J. Biol. Chem.* **246**, 2285–2290

Chaiken, I. M., Komoriya, A., Ohno, M. & Widmer, F. (1982) *Appl. Biochem. Biotechnol.* **7**, 385–399

Corradin, G. & Harbury, H. A. (1974) *Biochem. Biophys. Res. Commun.* **61**, 1400–1406

Crook, E. M., Mathias, A. P. & Rabin, B. R. (1960) *Biochem. J.* **74**, 234–238

Dykes, D. F., Creighton, T. & Sheppard, R. C. (1974) *Nature (London)* **247**, 202–203

Graf, L., Li, C. H., Cheng, C. H. K. & Jibson, M. D. **78**, 6135–6138

Graf, L., Li., C. H., Cheng, C. H. K. & Jibson, M. D. (1981) *Biochemistry* **20**, 7251–7258

Harris, D. E. & Offord, R. E. (1977) *Biochem. J.* **161**, 21–25

Homandberg, G. A. & Laskowski, M. (1979) *Biochemistry* **18**, 586–592

Homandberg, G. A., Matthis, J. A. & Laskowski, M. (1978) *Biochemistry* **17**, 5220–5227

Hunter, M. L. & Ludwig, M. J. (1962) *J. Am. Chem. Soc.* **84**, 3491–3504

Jacobs, E. E. & Sanadi, D. R. (1960) *J. Biol. Chem.* **235**, 531–534

Juillerat, M. & Homandberg, G. A. (1981) *Int. J. Pept. Protein Res.* **18**, 335–342

Juillerat, M., Parr, G. R. & Taniuchi, H. (1980) *J. Biol. Chem.* **255**, 845–853

Komoriya, A., Homandberg, G. A. & Chaiken, I. M. (1980) *Int. J. Pept. Protein Res.* **16**, 433–439

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Margoliash, E. & Smith, E. L. (1962) *J. Biol. Chem.* **237**, 2151–2160

Offord, R. E. (1966) *Nature (London)* **211**, 591–593

Offord, R. E. (1980) *Semisynthetic Proteins*, John Wiley and Sons, London

Offord, R. E. (1983) in *Peptides 1982 (Proc. Eur. Pept. Symp. 17th)* (Blaha, K. & Malon, P., eds.), pp. 31–42, Walter de Gruyter, Berlin

Proudfoot, A. E. I. & Wallace, C. J. A. (1983) in *Peptides 1982 (Proc. Eur. Pept. Symp. 17th)* (Blaha, K. & Malon, P., eds.), pp. 353–356, Walter de Gruyter, Berlin

Richards, F. M. & Wyckoff, H. W. (1971) *Enzymes 3rd Ed.* **4**, 647–746

Rose, K., Simona, M. G. & Offord, R. E. (1983) *Biochem. J.* **215**, 261–272

Sealock, R. W. & Laskowski, M., Jr. (1969) *Biochemistry* **1**, 3703–3710

Vogel, A. I. (1956) *A Text-Book of Practical Organic Chemistry*, 3rd edn., pp. 330–331, Longmans, Green and Co., London

Wallace, C. J. A. (1984) *Biochem. J.* **217**, 595–599

Wallace, C. J. A. & Harris, D. E. (1984) *Biochem. J.* **217**, 589–594

Westerhuis, L. W., Tesser, G. I. & Nivard, R. J. F. (1979) *Rec. Trav. Chim. Pays-Bas* **98**, 109–112