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Trifluoroacetylated Cytochrome *c**

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ABSTRACT: The lysine residues of horse heart cytochrome *c* can be trifluoroacetylated by reaction with ethyl thioltrifluoroacetate and the blocking groups subsequently removed by mild alkaline hydrolysis. The regenerated material displays full electron-transfer activity in the succinate oxidase system and moves on Amberlite CG-50 in the same manner as fraction I of unmodified preparations.

Trifluoroacetylated cytochrome *c* containing no amino groups detectable by dinitrophenylation or treatment with nitrous acid is without activity in the succinate oxidase system, but displays, over the range of pH 6–10, Soret and visible spectra essentially unchanged from those of nontrifluoroacetylated samples. By tryptic hydrolysis of the trifluoroacetylated protein, a heme peptide of 38 amino acid residues, representing approximately one-third of the cytochrome molecule, has been prepared.

Treatment of horse heart cytochrome *c* with acetic anhydride, under conditions leading to acylation of about half the lysine residues, has been reported to result in complete inactivation of the molecule (Minakami *et al.*, 1958; Takemori *et al.*, 1962). It is not known whether all groups other than lysine remained unmodified in these experiments, but the inference generally drawn, and consistent with various other observations, is that the loss of appreciable numbers of positively charged groups leads to disruption of essential structural features of the protein and impairs its binding to other components of the electron-transfer chain (*e.g.*, Margoliash, 1961, 1962).

Reaction with ethyl thioltrifluoroacetate (Schallenberg and Calvin, 1955) would thus be anticipated also to bring about loss of activity. This reaction is, however, of special interest in that trifluoroacetylamide linkages can be cleaved hydrolytically under conditions where peptide bonds remain intact, a circumstance which makes possible the use of trifluoroacetyl (TFA)[1] groups

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[1] Abbreviations used in this work: TFA, trifluoroacetyl; TFA-cytochrome *c*, trifluoroacetylated cytochrome *c* prepared as described.

as removable amine-blocking or protective agents. The procedure has found use principally in the field of peptide synthesis, but has been applied also to ribonuclease (Goldberger and Anfinsen, 1962), and, if practicable in the case of cytochrome *c*, would be a helpful addition to the techniques available for studies of the structure and function of this molecule. One of the more obvious possibilities would be its use in the attainment of greater selectivity in methods for peptide chain cleavage and the modification of functional groups.

It is shown in this report that the lysine residues of horse heart cytochrome *c* can apparently all be trifluoroacetylated and then reconverted to the free amino form with regeneration of activity. Some of the properties of the blocked and deblocked preparations are given, and use of the former in the preparation of a heme peptide of 38 amino acid residues is described.

Materials and Methods

Materials. Horse heart cytochrome *c* was obtained from Sigma Chemical Co. (Type III). Ethyl thioltrifluoroacetate was prepared as described by Hauptschein *et al.* (1952). Twice-crystallized trypsin (bovine pancreas) was a product of Worthington Biochemical Corp. L-(1-Tosylamido-2-phenyl)ethyl chloromethyl ketone (Schoellmann and Shaw, 1963) was obtained from California Biochemical Corp. ε-DNP-L-lysine was a gift of Dr. William Konigsberg. α-DNP-L-lysine was prepared by dinitrophenylation (Sanger, 1945) of ε-TFA-L-lysine (Schallenberg and Calvin, 1955), followed by hydrolysis with constant-boiling hydrochloric acid at 110° for 16 hr. Other DNP-amino acids were prepared as outlined by Fraenkel-Conrat *et al.* (1955) or were obtained from Mann Research Laboratories, Inc.

Amino Acid Analysis. Quantitative analyses were carried out with a Beckman-Spinco Model 120B automatic amino acid analyzer (Moore *et al.*, 1958; Spackman *et al.*, 1958). Samples were hydrolyzed with constant-boiling hydrochloric acid in evacuated ampoules at 110°. For DNP derivatives, the period of hydrolysis was 16 hr. For compounds other than DNP derivatives, it was 24 hr. Paper chromatography (Fraenkel-Conrat *et al.*, 1955) was used for qualitative analysis of DNP-amino acids.

Paper Electrophoresis. Electrophoretic mobilities of protein preparations were compared at a potential of 15 v/cm, using a pyridine acetate buffer (1 M pyridine) of pH 6.5.

Determination of Activity. Electron-transfer activity was estimated with the use of a rat liver succinate oxidase system (Schneider and Potter, 1943). Oxygen uptakes were followed manometrically under conditions specified previously (Hettinger and Harbury, 1964).

Spectrophotometry. Absorption spectra were recorded with a Bausch and Lomb Spectronic 505 spectrophotometer. A Thunberg type cuvet assembly was used (silica absorption cell, graded seal to upper vessel). Before reduction, samples were subjected to several cycles of evacuation and admission of helium. Sodium dithionite, tipped in from the side arm, served as the reducing agent.

Dinitrophenylation and Deamination. Reaction of proteins and amino acids with fluorodinitrobenzene was conducted in 67% ethanol essentially as described by Sanger (1945), except that protein concentrations were 25-fold lower and the initial ethanol-free samples were saturated with bicarbonate. Reaction with nitrous acid was carried out for 2 hr under the conditions developed by Van Slyke (1929).

Preparation of TFA-cytochrome c. The reaction was performed at room temperature. A mixture of 5 ml of ethyl thioltrifluoroacetate and 500 mg of horse heart cytochrome *c* in 20 ml of water was kept, with continuous stirring, at pH 10.0. Maintenance of pH was effected by the addition of 1 M potassium hydroxide, regulated by a Radiometer TTT1c titrator operated as a pH-Stat. After 50 min, 2 ml more of the ester was introduced. At the end of a total of 90 min, the pH was brought to 8.0 with hydrochloric acid, the mixture filtered, and the filtrate subjected to dialysis against 0.01 M phosphate buffer at pH 8.0, followed by dialysis against water.

The solution then was lyophilized and the product subjected to gel filtration at 0° on Sephadex G-75. Samples were applied in 0.02 M phosphate buffer, pH 7.5, to a column (2.5 × 50 cm) previously equilibrated with this buffer. Two well-separated fractions were obtained: a minor one, brown in color, and a slower moving, major component (about 85% of the material applied), red in color. This second fraction was dialyzed against water, lyophilized, and stored at −15°.

Preparation of Heme Peptide from TFA-cytochrome c. Hydrolysis of the TFA-cytochrome *c* was effected with trypsin pretreated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (Schoellmann and Shaw, 1963) to minimize chymotryptic activity (Carpenter and Kostka, 1964). A solution of 30 mg of TFA-cytochrome *c* and 0.3 mg of the treated trypsin in 4.0 ml of 0.1 M Tris buffer, pH 8.0, was kept at 25° for 6 hr. Some of the sample was taken for estimation of free α- and ε-amino groups. The remainder was applied to a column (0.8 × 10 cm) of TEAE-cellulose previously equilibrated with the same buffer, and then was eluted by the successive addition of 0.2, 0.3, and 0.4 M Tris buffer of pH 8.0. Approximately 60% of the heme-containing material, representing unreacted TFA-cytochrome *c*, remained at the top of the column. All of the rest was contained in the 0.4 M fraction, which was dialyzed against water and lyophilized.

Removal of TFA Groups. The trifluoroacetylamide linkage was hydrolyzed at pH 10.7. Samples of TFA-cytochrome *c* and TFA-heme peptide at a concentration of 1% were kept for 30 hr at 25° in 0.15 M potassium carbonate buffer. In the case of the protein, portions of the solution were removed at intervals and subjected to paper electrophoresis and dinitrophenylation. At the end of 30 hr the solutions were dialyzed against water and then lyophilized. Cytochrome *c* so obtained was chromatographed on Amberlite CG-50 as described by

TABLE I: Amino Acid Composition of Cytochrome *c*, TFA-Cytochrome *c*, and Heme Peptide from TFA-Cytochrome *c*.

Amino Acid	Residues/Mole of Protein or Peptide[a]			
	Cytochrome *c*[b]	TFA-Cytochrome *c*	Regenerated Cytochrome *c*	Heme Peptide[c]
Lysine	18.8 (19)	19.0	18.6	6.8 (7)
Histidine	3.1 (3)	3.1	3.0	3.0 (3)
Arginine	2.0 (2)	1.9	2.0	1.1 (1)
Aspartic acid	8.2 (8)	8.1	8.0	1.9 (2)
Threonine	9.5 (10)	9.4	9.3	2.0 (2)
Serine	0 (0)	0	0.4	0.1 (0)
Glutamic acid	11.9 (12)	11.8	12.1	4.2 (4)
Proline	4.0 (4)	4.1	3.9	1.2 (1)
Glycine	12.3 (12)	11.6	12.1	6.7 (7)
Alanine	6.2 (6)	5.8	6.1	1.0 (1)
Half-cystine[d]	1.3 (2)	1.3	1.8	0.8 (2)
Valine	2.8 (3)	2.9	3.0	2.9 (3)
Methionine	1.9 (2)	2.0	1.9	0 (0)
Isoleucine	5.6 (6)	6.1	5.9	1.1 (1)
Leucine	6.0 (6)	6.0	5.9	2.0 (2)
Tyrosine	3.8 (4)	3.7	3.7	0 (0)
Phenylalanine	3.9 (4)	3.9	4.0	2.0 (2)

[a] The measured values were adjusted to residues/mole of protein or peptide by use of a constant multiplying factor, obtained from the following amino acids upon assumption of the compositions: protein, $Arg_2Ala_6Asp_8Glu_{12}Gly_{12}Leu_6$; peptide, $Arg_1Ala_1Asp_2Glu_4Gly_7Leu_2$. No corrections were made for loss on hydrolysis. [b] Literature values (Margoliash *et al.*, 1961) in parentheses. [c] Values for residues 1–38 of cytochrome *c* in parentheses. [d] Thioether bridges not cleaved prior to hydrolysis.

Margoliash and Lustgarten (1962). No material corresponding to fraction II was seen.

For purposes of comparison, TFA-cytochrome *c* was also hydrolyzed under more vigorous conditions, using 1 M piperidine at 0° (Goldberger and Anfinsen, 1962), and 0.02 M potassium hydroxide and concentrated ammonium hydroxide at 25° (Schallenberg and Calvin, 1955).

Results

Unmodified cytochrome *c*, TFA-cytochrome *c*, and cytochrome *c* regenerated from TFA-cytochrome *c* all yielded essentially the same amino acid analysis (Table I).

All ε-amino groups in the trifluoroacetylated protein appeared to be masked. Dinitrophenylation of unmodified cytochrome *c* resulted in a lysine analysis of 0.5 residue/mole of protein and a content of ε-DNP-lysine of 18.6 residues/mole. Treatment of TFA-cytochrome *c* with fluorodinitrobenzene was, in contrast, without effect on the lysine content, and no ε-DNP-lysine was formed. Likewise, cytochrome *c* modified with nitrous acid gave a lysine value of 0.4 residue/mole of protein, whereas TFA-cytochrome *c* subjected to the same treatment yielded an analysis of 18.7 lysine residues/mole. The data are summarized in Table II.

Further evidence was obtained upon dinitrophenylation of a sample of TFA-cytochrome *c* partially cleaved with trypsin. Analysis of the dinitrophenylated mixture indicated the presence of 18.3 residues of lysine/mole of initial protein, and no ε-DNP-lysine was found[2] (Table II).

Paper chromatography of the DNP-amino acids formed upon acid hydrolysis of the dinitrophenylated tryptic digest led to observation of but one α-DNP-derivative, α-DNP-lysine, and, consistent with this, but a single heme peptide was obtained as a result of the limited tryptic action. Its composition corresponded to residues 1–38 of the intact cytochrome molecule (Table I). Cleavage thus occurred between arginine 38 and lysine 39. The fact that no α-DNP-glutamic acid was observed suggests there may have been little or no breakage at the other of the two arginine residues of the protein, arginine 91. If so, a simple procedure may be at hand for splitting cytochrome *c* into just two components, comprising approximately one-third and two-thirds of the parent molecule, respectively.

The Soret and visible absorption spectra of TFA-cytochrome *c*, measured over the range pH 6–10, were found to be essentially identical with those of un-

[2] At the concentration of material applied to the analyzer in this experiment, ε-DNP-lysine at a level less than 0.2 residue/mole of protein would have escaped detection.

TABLE II: Results of Treatment with Fluorodinitrobenzene and Nitrous Acid.

Sample	Residues/Mole of Protein[a] Lysine	His-tidine	Argi-nine[b]	ε-DNP-Lysine
Fluorodinitrobenzene				
Cytochrome *c*	0.5	0.2	2.0	18.6
TFA-cytochrome *c*	19.1	0.2	2.0	0
TFA-cytochrome *c*, tryptic hydrolysate	18.3	0.2	2.0	0[c]
Nitrous acid				
Cytochrome *c*	0.4	2.9	2.0	
TFA-cytochrome *c*	18.7	3.2	2.0	

[a] Average of two analyses each. [b] Taken as reference. [c] See footnote 2.

modified cytochrome *c*. Ferro-TFA-cytochrome *c* remained a hemochrome, and ferri-TFA-cytochrome *c* a hemichrome, throughout the range covered. Below pH 6, there is a progressive diminution in solubility, and, in more alkaline solution, increasingly rapid deacylation takes place.

TFA-cytochrome *c* did not, on the other hand, resemble the unmodified protein in terms of activity in the succinate oxidase system. There was no stimulation of oxygen uptake.

Removal of the TFA groups was effected in several ways. Treatment with 1 M piperidine, concentrated ammonium hydroxide, or 0.02 M potassium hydroxide led to the most rapid liberation of free ε-amino groups. It seemed desirable, though, to use the gentlest conditions practicable, and the procedure adopted consisted of slower hydrolysis at pH 10.7 in carbonate buffer. After 16 hr under these conditions the protein exhibited the same electrophoretic mobility as did unmodified material, but analysis indicated that 1.3 lysine residues still were refractory to dinitrophenylation. By the end of 30 hr, however, the content of lysine after dinitrophenylation and acid hydrolysis agreed to within 0.2 residue/mole with the corresponding value for unmodified cytochrome *c*. A summary is given in Table III.

Upon chromatography of the deblocked material on Amberlite CG-50 no fraction II (Margoliash and Lustgarten, 1962) was found, and the eluted protein displayed full electron-transfer activity in the succinate oxidase system (Table IV).

TABLE III: Regeneration of Amino Groups.

Sample Dinitrophenylated	Residues/Mole of Protein[a] Lysine	His-tidine	Argi-nine[b]	ε-DNP-lysine
Unmodified cytochrome c	0.5	0.2	2.0	18.6
Regenerated cytochrome c				
Carbonate buffer, pH 10.7, 25°, 8 hr	2.9	0.1	2.0	15.9
Carbonate buffer, pH 10.7, 25°, 16 hr	1.3	0.1	2.0	17.7
Carbonate buffer, pH 10.7, 25°, 24 hr	0.9	0.1	2.0	17.9
Carbonate buffer, pH 10.7, 25°, 30 hr	0.7	0.1	2.0	18.5
Potassium hydroxide, 0.02 M, 25°, 6 hr	1.0	0.1	2.0	18.3
Potassium hydroxide, 0.02 M, 25°, 24 hr	0.6	0.2	2.0	18.2
Piperidine, 1 M, 0°, 6 hr	0.6	0.1	2.0	18.6
Ammonium hydroxide, concentrated, 25°, 6 hr	1.1	0.3	2.0	18.1
Ammonium hydroxide, concentrated, 25°, 26 hr	0.7	0.2	2.0	18.3

[a] Average of two analyses each for unmodified cytochrome *c* and cytochrome *c* regenerated in carbonate buffer. Others, single analyses. [b] Taken as reference.

TABLE IV: Electron-Transfer Activity in the Succinate Oxidase System.

Sample	Oxygen Uptake[a] (μl min^{-1}) 1.0 μM	4.0 μM	7.0 μM
Unmodified cytochrome *c*	1.5	4.3	5.0
TFA-cytochrome *c*	0	0	0
Regenerated cytochrome *c*	1.5	4.1	5.1

[a] Corrected for blank.

Discussion

The results of dinitrophenylation, reaction with nitrous acid, and tryptic hydrolysis all suggest that the TFA-cytochrome *c* contained no free ε-amino groups. Whereas the lysine residues of unmodified cytochrome *c* could be converted almost entirely to the ε-DNP and deaminated derivatives, none of the lysine residues of TFA-cytochrome *c* could be so modified, and the fact that two different forms of treatment, involving widely different conditions, yielded fully concordant results renders it unlikely that some of the amino groups in TFA-cytochrome *c* were protected simply by conformational factors. This is a conclusion supported

further by the observation that no ε-DNP-lysine was obtained upon dinitrophenylation of a solution of TFA-cytochrome *c* partially hydrolyzed with trypsin, and the finding, upon fractionation of this hydrolysate, of but a single heme peptide, formed by cleavage after an arginine residue.

Removal of the TFA groups could be effected under mild conditions, and, by the criteria thus far applied, led to regeneration of cytochrome *c* in its original form. Movement on Amberlite CG-50 corresponded to that of fraction I of the unmodified protein (Margoliash and Lustgarten, 1962), the reactivity toward fluorodinitrobenzene and nitrous acid reverted to that seen before trifluoroacetylation, and full electron-transfer activity in the succinate oxidase system was recovered.

The ability to mask the amino groups reversibly is potentially useful in a variety of applications. An illustration is the present preparation of a heme peptide of 38 residues, sought in connection with comparative studies of a selected series of such derivatives (Harbury and Loach, 1959, 1960a,b; Y. P. Myer and H. A. Harbury, in preparation), and the first of this group to contain all three histidine residues of the protein.

The tryptic cleavage of TFA-cytochrome *c* used to obtain this peptide involved hydrolysis at arginine residue 38, and, as would be expected from the amino acid sequence, resulted in the formation of N-terminal lysine. Further cleavage of the protein, at its second arginine residue (position 91), would be anticipated to yield N-terminal glutamic acid, but, under the conditions used, none was found. There may thus have been a significant difference in the rate of reaction at the two sites. Should further work confirm this, the way might be open for recombination experiments between a heme peptide and a nonheme peptide comprising jointly the full amino acid sequence of the intact protein.

The lack of effect of trifluoroacetylation on the Soret and visible spectra of the molecule suggests that the modification procedure was unattended by change in the nature of the central coordination complex. The groups bound to the heme iron in unmodified cytochrome *c* apparently remain so bound in TFA-cytochrome *c* at neutral pH. Similar results were obtained previously with the fully guanidinated derivative (Hettinger and Harbury, 1964, 1965), and the simplest interpretation continues to be that, at physiologic pH, the groups of the protein acting as ligands do not include an amino group.

It seems likely that the primary binding function of the lysine residues is related to the interaction of cytochrome *c* with other components of the electron-transfer chain, and it will be of interest to see in greater detail in just what manner the activity varies with the numbers and distribution of ε-ammonium groups available. Trifluoroacetylation, because of its reversibility and the favorable properties of the derivatives formed, would appear to offer special advantages for such studies.

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