III. 2. THE ROLE OF SULFUR IN CYTOCHROME c

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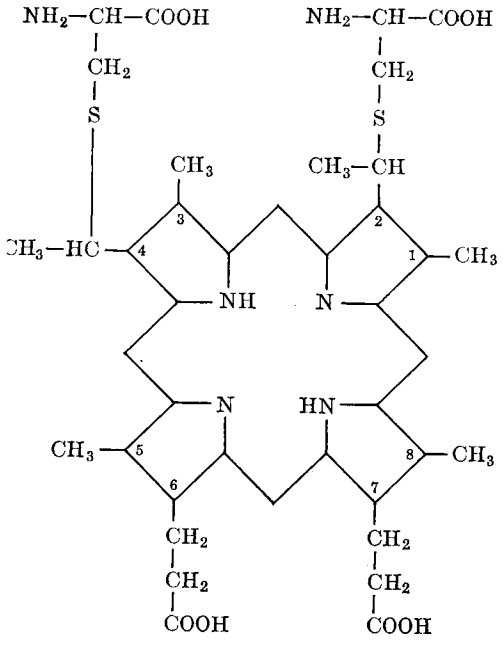
I. The Thioether Bridges in Cytochrome c

When acid acetone is added to hemoproteins such as catalase, peroxidase, hemoglobin, myoglobin, or to cytochromes a and b, the hematin prosthetic group is detached from the protein moiety. In cytochrome c, however, porphyrin and protein will not be dissociated by this treatment. Theorell (*1*) was the first to demonstrate that sulfur was involved in establishing this rather more stable linkage between apoprotein and prosthetic group.

Early estimations of the sulfur content in cytochrome c had led to the view that there were six atoms of sulfur per molecule (*2*). More recent determinations, however, which have been carried out by Paléus (*3*) on preparations of cytochrome c purified by ion exchange chromatography, have provided good evidence for the presence of four atoms of sulfur only. Two of them belong to methionine residues (*4*) and have so far not attracted further attention. The other two, however, are outstanding in remaining attached to the pigment moiety of the molecule even when the hemoprotein is degraded by acid hydrolysis. Theorell in Sweden and Zeile and Meyer (*5*) in Germany were able to isolate and to purify, from acid hydrolysates of cytochrome c, a compound designated as porphyrin c, to which the structure represented by Formula I was ascribed (*6, 7*). Porphyrin c may be thought of as being built up of one protoporphyrin molecule and two cysteine molecules the thiol groups of which have been added to the vinyl side chains of the porphyrin. In the intact cytochrome c molecule,

of course, the prosthetic group contains iron as the central atom, and the two porphyrin-linked cysteine residues are incorporated into the protein moiety of the hemoprotein through peptide bonds.



Porphyrin c
(I)

An important further step was taken when Paul (*8*) succeeded in breaking the two thioether linkages by treating cytochrome c with silver salts in weakly acidic solution. He thereby obtained, on the one hand, the apoprotein free from pigment and, on the other hand, the pigment in the form of optically active hematohemin (*9*).

II. The Amino Acids Adjoining the Porphyrin-Linked Cysteine Residues

The facts reviewed above were the starting point for the more recent investigations which aimed at gaining a somewhat more detailed picture of the linkage between apoprotein and prosthetic group and of the nature of the "active center" of cytochrome c. In these experiments the hematin prosthetic group has been used as a natural color mark indicating that region of the protein which contains the two cysteine residues.

To begin with, horse and beef cytochromes c were hydrolyzed partially with dilute sulfuric acid at 100°, so as to obtain, besides free porphyrin c,

porphyrin c peptides, i.e. compounds in which the porphyrin-linked cysteine residues of cytochrome c were still connected with other amino acids (*10, 11*). These colored products could be separated from free amino acids and porphyrin-free peptides which were copiously present in the hydrolyzate, by adsorption on talc at a pH of 3 to 4. After elution from the adsorbent, with the help of aqueous ethanol-containing ammonia, the porphyrin c peptides were subjected to the treatment with silver sulfate in acetic acid which Paul (*8*) had successfully applied to intact cytochrome c. The thioether bridges were readily broken and peptides of cysteine were set free. As cysteine peptides could not easily be separated from each other by chromatography, they were first oxidized with performic acid. The mixture of cysteic acid peptides obtained was resolved into its components by ionophoresis and by paper chromatography. The cysteic acid-containing peptides (II)

Lys- $CySO_3H$ Glu-[$CySO_3H$, His]
Lys-[$CySO_3H$, Ala] $CySO_3H$-His
$CySO_3H$-Ala

(II)

which were identified in the course of an investigation of beef cytochrome c, permitted the conclusion that one of the two cysteine residues in cytochrome c was flanked by lysine and alanine, and the other one by glutamic acid and histidine.

III. Structure and Properties of Hemopeptides Obtained from Cytochrome c by Enzymatic Degradation

More could be learnt about the protein region neighboring the prosthetic group by experiments involving an enzymatic degradation of the hemoprotein. Digestion of cytochrome c with pepsin (*12*) yielded, besides other peptides, a red-colored split product of low molecular weight which had an absorption spectrum in the visible wave length range very similar to that of its mother compound and in which the hematin prosthetic group was present intact. A similar degradation product of cytochrome c had previously been obtained by Tsou (*13*). His "pepsin-modified cytochrome c," however, had not been pure enough for an investigation of its amino acid sequence. In order to arrive at a homogeneous preparation, a series of purification steps were carried out: precipitations with ammonium sulfate and trichloroacetic acid, isoelectric agglutination at pH 5, and partition chromatography on columns of Hyflo Supercel using butanol-acetic acid as the developing agent.

An amino acid analysis by the method of Moore and Stein (*14*) showed that this peptic "hemopeptide" contained one residue each of threonine,

alanine, histidine, and lysine, two valine residues and three glutamic acids, apart from the two porphyrin-linked cysteine residues. The eleven building stones proved to be arranged in one single polypeptide chain and not, as might have been expected, in two chains, each of them comprising one cysteine. With the help of Sanger's DNP-technique (*15*) it was established that there was present only one N-terminal residue, namely valine, and that this was followed by glutamic acid in the polypeptide chain. On incubation with trypsin, a tripeptide, Val-Glu(NH_2)-Lys, was split off from the rest of the hemopeptide. This tripeptide was also derived from the amino-terminal end of the chain.

In order to work out the whole amino acid sequence the hemopeptide was first split into its hematin and polypeptide moieties, again using Paul's silver salt method, followed by an oxidation of the cysteine residues with performic acid. Thereby a colorless porphyrin-free undecapeptide was obtained which had two cysteic acid residues instead of two cysteine residues.

When the undecapeptide was acted upon by the bacterial enzyme subtilisin (*16*), mainly five short peptides were produced: 4 dipeptides, Val-Glu(NH_2), [Lys,$CySO_3H$], Ala-Glu(NH_2), and [$CySO_3H$,His], and the tripeptide Thr-[Val,Glu]. When, on the other hand, the undecapeptide was subjected to a partial hydrolysis with concentrated hydrochloric acid, a complex mixture of degradation products was obtained among which the peptides shown (III) were identified:

Val-Glu	[$CySO_3H$,	Ala,	Glu]	$CySO_3H$-His	
Val-[Glu,	Lys]	Ala-	Glu		Thr-[Val, Glu]
	Lys-$CySO_3H$		Glu-	$CySO_3H$	
	$CySO_3H$-	Ala	Glu-	[$CySO_3H$, His]	

(III)

These results indicate that the sequence of eleven amino acid residues must be as shown (IV).

Val-Glu(NH_2)-Lys-$CySO_3H$-Ala-Glu(NH_2)-$CySO_3H$-His-Thr-[Val, Glu]

(IV)

The action of trypsin upon cytochrome c (*11, 17*) yielded a hemopeptide which was rather similar to the one obtained by peptic digestion. All the amino acids were contained in a single polypeptide chain as, after splitting with silver sulfate in acetic acid solution and oxidation, a single porphyrin-free polypeptide was recovered. The DNP-method showed one cysteic acid residue to be the N-terminal amino acid, whereas another cysteic acid residue present in the chain was nonterminal. From a series of small peptides (V) identified in a partial hydrolyzate of the porphyrin-

free polypeptide,

$CySO_3H$- Ala $CySO_3H$ - His
$CySO_3H$-[Ala, Glu] Thr-[Val, Glu]
Ala- Glu Thr-[Val, Glu, Lys]
Glu-$CySO_3H$ Glu - Lys
Glu-[$CySO_3H$, His]

(V)

the amino acid sequence (VI)

$CySO_3H$-Ala-Glu(NH_2)-$CySO_3H$-His-Thr-Val-Glu-Lys

(VI)

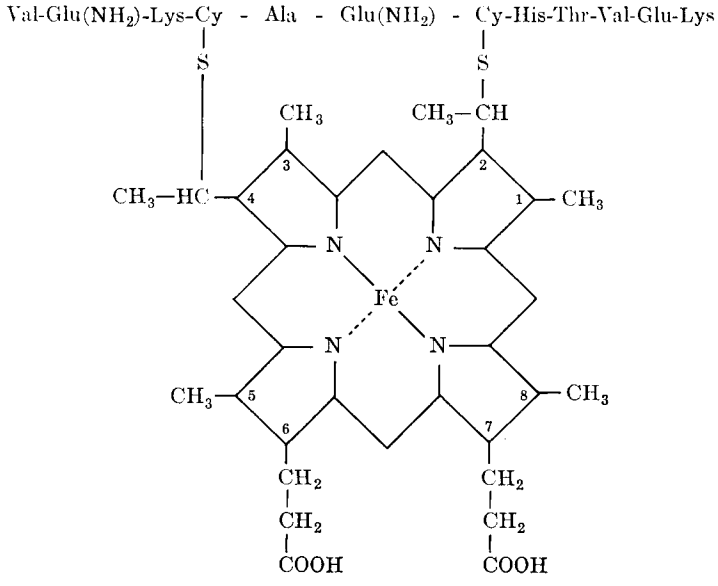
could be deduced.

At the N-terminal end this nonapeptide sequence is shorter than the sequence in the peptic degradation by three amino acid residues, whereas on the C-terminal side it is longer by one (lysine) residue. The way in which the nonapeptide sequence has been broken out of the protein moiety of cytochrome c is in perfect agreement with the known enzymatic specificity of trypsin. Trypsin splits exclusively peptide bonds involving the carboxyl groups of lysine or arginine residues (*18, 19*).

The two cysteic acid residues present in the tryptic nonapeptide and the peptic undecapeptide sequences have arisen, through performic acid oxidation, from the two porphyrin-linked cysteine residues of cytochrome c. It may, thus, be summarized that one single polypeptide chain contains both cysteine residues which provide the stable sulfur bridges between the protein moiety and the prosthetic group in cytochrome c, and that these two cysteine residues are separated from each other by two other amino acid residues.

There are two possible ways of attaching the heme part to the peptide chain by two thioether bonds (*17*). The alternatives differ in which one of the two cysteine residues will be added to the vinyl side chain in position 2 of the porphyrin ring, and which one to the vinyl side chain in position 4. The alternative represented in Formula VII is the more probable, for steric reasons (*20*).

The finding that in the amino acid sequence a histidine residue is adjacent to one of the two cysteine residues is remarkable in view of the important role which histidine residues have for many years been supposed to play in cytochrome c. In its oxidized as well as in its reduced form, cytochrome c exhibits a characteristic hemochromogen spectrum, i.e. the spectrum of an iron porphyrin compound in which the central iron atom is linked not only with the 4 pyrrole nitrogen atoms of the porphyrin but, in addition, with one or two more nitrogen-containing groups. Theorell



(VII)

and Åkeson (*2*) concluded, as a result of their ingenious spectrophotometric, titrimetric, and magnetic investigations, that at least one and presumably both of the "heme-linked" nitrogenous groups were imidazole groups of histidine residues. [There are, on the whole, three histidine residues per molecule of cytochrome c (*2, 20, 21*).]

From our finding that a histidine residue adjoined the porphyrin-linked cysteine residues, it appeared rather likely that its imidazole group was involved in bringing about the hemochromogen nature of cytochrome c (*10, 17*). By building steric models of the hemopeptide structure given in Formula VII, Ehrenberg and Theorell (*20*) were able to demonstrate that the formation of two thioether bonds could be readily effected if the polypeptide chain which contains the two cysteine residues suitably spaced by two other amino acid residues was in the form of an α-helix. Furthermore, if the polypeptide chain was turned into an α-helix of the left-hand type, the histidine residue was taking a position utterly favorable for an attachment of an imidazole nitrogen atom to the hematin iron. Thus, the fact that two cysteine residues, separated from one another by two intermediary amino acid residues, are attached to the vinyl side chains of a protohemin molecule will force the polypeptide chain into a rigid α-helix the axis of which is parallel to the plane of the heme disk. This secondary structure of the chain will hold the histidine imidazole group rather firmly to the heme iron so as to effect hemochromogen formation.

IV. Species Specificity of Amino Acid Sequence and Constancy of Basic Structure in Cytochrome c

Assuming that characteristic for cytochrome c was the structure involving two porphyrin-linked cysteine residues separated from each other by two other residues and the adjoining position of a heme-iron-bound histidine residue, it was to be expected that this pattern of amino acid residues should be observed in all compounds with the spectroscopic and enzymatic properties of cytochrome c, irrelevant of their origin. Other amino acid residues in the protein moiety of the hemoprotein may be supposed to be exchangeable; the basic pattern, however, should be constant.

With this working hypothesis in mind, cytochrome c was isolated from different sources, not only from the hearts of mammals, but also from fish, birds, insects, and yeast. The hemoproteins were degraded with the help of either trypsin or pepsin, and the resulting hemopeptides, after careful purification, were compared with the corresponding hemopeptides obtained from cattle cytochrome c (Table I). In two mammalian cytochromes

TABLE I

THE AMINO ACID SEQUENCE IN HEMOPEPTIDES OBTAINED FROM CYTOCHROMES c OF DIFFERENT ORIGIN BY PEPTIC DEGRADATION

Beef Horse Pig	. . .Val-Glu(NH_2)-Lys-CyS-Ala-Glu(NH_2)-CyS-His-Thr-Val-Glu. . .
Chicken	. . .Val-Glu(NH_2)-Lys-CyS-Ser-Glu(NH_2)-CyS-His-Thr-[Val, Glu]. . .
Salmon	. . .Val-Glu(NH_2)-Lys-CyS-Ala-Glu(NH_2)-CyS-His-Thr-[Val, Glu]. . .
Silkworm	. . .Val-Glu(NH_2)-Arg-CyS-Ala-Glu(NH_2)-CyS-His-Thr-[Val, Glu]. . .
Bakers' yeast	. . .Phe-Lys-Thr-Arg-CyS-Glu-Leu-CyS-His-Thr-[Val, Glu]. . .

(horse and pig) the sequence of amino acids in the neighborhood of the prosthetic group proved to be identical with the one established in beef cytochrome c (*11*). In salmon, the same sequence was again found to occur (*12*). In chicken cytochrome c, however, a serine residue was shown to be present where in mammalian and in fish material an alanine had been located (*12*). This replacement is especially interesting as it has occurred in that part of the polypeptide chain which is intercalated between the two cysteine residues. Silkworm cytochrome c (*22*), on the other hand, differs from the others in possessing an arginine residue instead of a lysine residue in the position adjoining one of the cysteine residues. More marked deviations were observed in a yeast cytochrome c (*23*). From bakers' yeast it was possible to extract two hemoproteins possessing cytochrome

c activity and to separate them from each other by ion exchange chromatography. So far, only one of them—the one which is more abundant in the yeast extracts—has been investigated in detail. In this yeast cytochrome, both amino acid residues situated between the thioether-forming cysteine residues, and quite a few others on the left-hand side of the sequence, are different from and partly unrelated to the respective ones in animal cytochromes. Here again, however, the presence of two cysteine residues within the same polypeptide chain, the constant distance between them, and the adjoining position of a histidine residue proved to be invariable. As recent experiments have shown (Paléus and Tuppy, unpublished) this holds true even for a cytochrome c-like pigment which has been isolated by Kamen and Vernon (*24*) from the photosynthetic bacterium *Rhodospirillum rubrum*, though this bacterial hemoprotein exhibits an enzymatic specificity somewhat different from that of the animal and yeast cytochromes (*25*). The sequence of amino acids, as far as it has been worked out in a tryptic degradation product of *Rhodospirillum* cytochrome c, appears to be (VIII).

CyS-Leu-Ala-CyS-His-Thr-Phe-[Asp, Glu, Gly, Ala, Asp]-Lys
(VIII)

V. The Tagging of Colored Residues to SH Groups in Proteins through Formation of Thioether Bridges

The natural occurrence of stable thioether linkages joining a protein component and a nonprotein prosthetic group is a feature confined to cytochrome c and, presumably, to cytochrome f (*26*). Experimentally, however, the thiol groups of cysteine residues occurring in proteins may be easily induced to form thioether bridges by reactions with compounds containing an activated double bond such as, for instance, derivatives of maleimide. There is a close formal analogy between the attachment of the SH groups of the cysteine residues in the polypeptide chain of cytochrome c with the vinyl side chains of a protohemin molecule, on the one hand, and the reaction of protein SH groups with *N*-ethylmaleimide, on the other hand, which has been widely used in protein and enzyme chemistry since its introduction in 1949 by Friedmann *et al.* (*27*). With the help of colored maleimide derivatives it should be possible to mark polypeptide regions adjoining cysteine residues in SH proteins. For this purpose, the yellow-colored *N*-[4-dimethylamino-3,5-dinitrophenyl]-maleimide has recently been synthesized and found to be a useful reagent (Witter and Tuppy, unpublished). After reacting it with a SH protein the latter can be degraded proteolytically and yellow peptide derivatives can be isolated. Unfortunately, it has not been possible to split, with silver salts, the thio-

ether bridge present in cysteine adducts of N-substituted maleimides. This failure shows that the analogy with the thioether bridges in cytochrome c must not be pushed too far, but this will not seriously limit the applicability of the reagent for structural investigations.

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Discussion

RIGGS: You mentioned that this peptide combines with or transfers oxygen. Is this reversible, and if so, to what extent?

TUPPY: It acts as an oxidase; it is autoxidizable and it strongly catalyses the oxidation of compounds such as ascorbic acid. With hydrogen peroxide and with benzidine or mesidine as substrates, the hemopeptide shows peroxidatic activity.

HAUROWITZ: I would like to ask whether you can exclude some secondary combination of heme with peptides. I am asking this because we wanted long ago to isolate heme by digesting hemoglobin with trypsin. If you digest hemoglobin with tryp-

sin completely and then acidify, you get what we called a hemin proteose with some peptide chains. So there must be some attachment of the peptide chains, perhaps peptide bond formation. You may know more about that.

TUPPY: In the case of cytochrome c we are fortunate to have a very clear situation. First of all, we are able to break down cytochrome c 100% with the help of trypsin which is not the case with hemoglobin where you always get a "core" resistant to hydrolysis. Secondly, the same kind of porphyrin-peptide linkage is found after acid hydrolysis and after enzymatic hydrolysis. This makes it appear very unlikely that the structure found is the result of a secondary reaction occurring, but of course one can never be quite sure.

KAUZMANN: We have made some studies on the denaturation of cytochrome c in urea and guanidine, and have found that 10 *M* urea has no effect on cytochrome c, but that if the heme group is removed with silver, it is very extensively unfolded in 10 *M* urea. So the cross-bracing provided by the cystines and the heme is important to the integrity of the native molecule.

FRIDOVICH: Do you have any notion with the iron completely in a cage, as it were, how electrons are transferred to and from it?

TUPPY: I have no clear idea. There is the possibility of the electrons passing by way of the imidazole groups of the histidine residues. This is, in my opinion, a reasonable hypothesis, and indeed, the only one I can conceive of.

BOYER: Dr. George has an interesting suggestion in this regard, that sulfur might actually be involved in the transfer of electrons. I am sorry he cannot be here to expound a little more on it.

JENSEN: At the University of Chicago, as some of you may know, Professor Taube in the chemistry department has done a lot of work on the mechanism of oxidation of one complexed metal ion by another. He finds very clearly that an anion serves to transfer the electron. One can think of a carbonate anion or a chloride anion in between the two cations with the electron passed from the reducing agent to the oxidizing agent through the inorganic anion. One can get some very interesting results in these experiments. Substances like maleic acid are especially good transfer ions because being longer they do not require that the two complex cations come so close together. Therefore, the rate of oxidation of one cation by the other is very much faster in the presence of such anions. We are intrigued with the possibility that phosphate is a transfer anion, ligand as Prof. Taube calls it, in the transfer of the electrons from one cytochrome to the other, and that this transfer is somehow associated with activation of the phosphate for oxidative phosphorylation.

KLOTZ: Except in this case of cytochrome, as I understand it, all six positions of the iron are taken. You would have to use one of the histidines in place of one of the anions you mention.

JENSEN: Exchange is possible because these positions should be mobile.

BOYER: That is an intriguing suggestion but still without supporting evidence. We made such a suggestion in an article in *Nature* in 1954. We have looked for exchanges of water oxygen with phosphate oxygen in model systems that might reveal temporary formation of such complexes followed by a cleavage of an O—P bond. So far we have found none, but this does not rule out the possibilities.

MAZUR: There is another possibility of transfer of electrons, and that is the one which occurs with an organic compound containing a system of alternate double bonds. This can take place among the quinones which have a tendency to polymerize. The compound acts as if it were a metal, as if it were conducting electrons. In the

case of a porphyrin, you can, with a little effort, show a system of conjugated double bonds. This may be the way you get your electron from ion to ion.

KLOTZ: In this case we are getting to the whole question of energy transfer and this type of concept has been suggested for polypeptides in general. You have Dr. Gergely here who has made calculations on this problem. I think we cannot get into the whole question of energy transfer right now.