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The Iron-protein Bonds in Cytochrome c

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Theorell and Åkeson suggested [1] that imidazole groups of histidine residues constitute the heme-linked groups in cytochrome c. The experimental background for this theory was in short as follows:

Amino acid analyses revealed that cytochrome c contained three histidine residues per molecule. Its titration curve showed that two equivalents of base were consumed per mole in the region pH 5.5—8.5, where the histidine imidazoles are normally titrated. A study of the heat of ionization disclosed, however, that other groups in cytochrome c than the imidazoles were also titrated in this pH range. In fact, only one imidazole group was free to combine with an acid or base, while the other two were somehow blocked. This was most easily explained by assuming linkages to the iron atom. Spectrophotometrically five different types of ferricytochrome (I—V, I being the most acid) could be distinguished, the pK values for the transitions being 0.42, 2.50, 9.35, and 12.76 respectively. With increasing acidity of the solution the histidine-iron bonds, present in type III, were released at pH 2.50 and 0.42 and substituted by protons. These low pK values for the ionizations of imidazole groups were explained as due to the influence of the iron atom. The dissociations in the alkaline region were attributed to the imino groups of the imidazole rings. Accordingly two pK values were found (9.35 and 9.85), only the former being optically operable. In agreement herewith ferricytochrome consumed two equivalents more per mole than ferrocytochrome did between pH 8.5 and 10.5. The transformation from type IV to type V appeared at higher alkalinity, where one of the histidine — iron bonds was displaced by a hydroxyl group. Ferricytochrome V and cyanide combine in the ratio 1 : with $K_{app.} = 6.16 \times 10^{-4}$ moles per liter *. Types III—V are diamagnetic, while the ionic bonds between the pyrrols and the iron in types I and II render these types paramagnetic.

* Unpublished experiment by K. G. P.

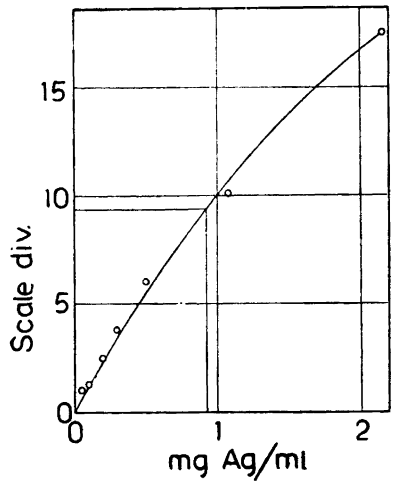

Fig. 1. Determination of silver in the protein moiety of cytochrome c. "Scale div." = difference in galvanometer deflections when the cuvette was filled with 1) water 2) the solution to be examined. Calibration curve made on silver nitrate solutions of given concentrations. The curve is drawn to fit the values. The protein solution, concentrated to 5.0 mg N/ml, gave 9.3 scale divisions, which is equal to 0.93 mg Ag/ml.

Since it is now possible to compare intact cytochrome c with its protein moiety [2], it was of interest to investigate if results from such a comparison were compatible with the above-mentioned theory.

EXPERIMENTS

Cytochrome c of the preparation with 0.424 % iron described in next paper [3] was used for the titration experiments in this paper. The fee prrotein was prepared by treatment of the cytochrome in the usual way [2] with silver sulphate and acetic acid (60°, 90 min.). The protein was then precipitated with ice-cold acid acetone, dissolved in water,reprecipitated, and dialyzed for four days against glass-distilled water. Electrodialysis was tried but found unsuitable, since the protein coagulated irreversibly at the cathode membrane. After the dialysis the clear solution was concentrated by freeze-drying and analyzed.

Electrophoretical examination showed uniform cathodical migration with the velocity 2.55×10^{-5} cm^2 × volt^{-1} × sec.$^{-1}$ (descending boundary, 1 % solution in acetate buffer pH 4.94, ionic strength 0.1).

The stock solution contained 1.998 mg of nitrogen per ml. The cytochrome preparation had been found to possess 145 nitrogen atoms per iron. After the removal of the prosthetic group from the molecule, the nitrogen content thus corresponded to 1.012×10^{-6} moles of the protein moiety per ml.

The silver content of the protein was of a special interest (*cf.* the discussion). Potentiometrical titrations of the ash, dissolved in nitric acid, with chloride gave inconsistent results, but by means of x-ray absorption measurements [4] the value 3.4 silver atoms per 141 nitrogen atoms was found * (Fig. 1). Because of the carbon and the nitrogen of the protein this value is slightly lower than the true value, which is estimated as 3.5—3.6.

A number of titration curves were made on the native cytochrome c and on its protein component. The latter was found to precipitate slightly below pH 5, and the titrations

* The author expresses his thanks to Docent Arne Engström for making the silver determination.

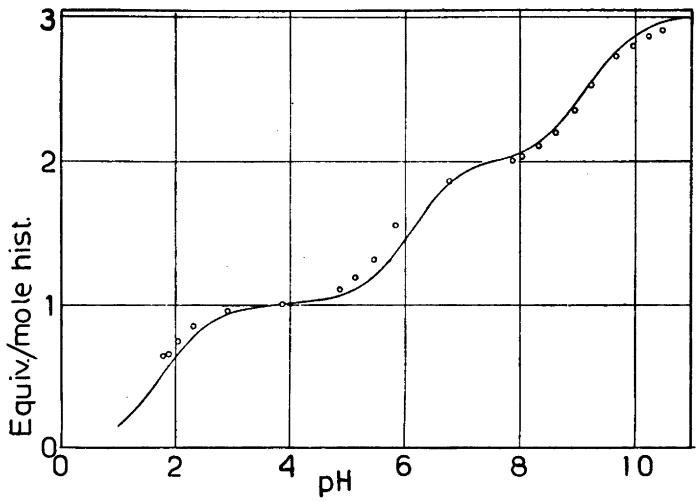

Fig. 2. Titration curve of histidine in glycerol-water 1 : 1 (O). The curve is calculated from pK values for histidine in water (Greenstein, J. P. J. Biol. Chem. 93 (1931) 479), the values being 1.77, 6.10, and 9.18 respectively.

were therefore carried out in water-glycerol. The effect of glycerol on the magnitude of the dissociation constants was studied on histidine (Fig. 2). In spite of the glycerol the protein began to flocculate at about pH 8.5, and consequently no reliable results could be obtained at higher alkalinity than pH 8. Those protein titrations, which were made in pure water between pH 1.7 and 4.5, agreed within this region fairly well with those made in glycerol-water.

One pair of titration curves of cytochrome c and its protein moiety in glycerol-water are given in Fig. 3. The results were reproduced in other experiments. The stock solution of cytochrome c was diluted with water to give a concentration of 1.997 mg of nitrogen per ml (micro-Kjeldahl determination on the diluted solution). The protein solution was taken directly for the titrations. To obtain equivalents of base combining with one mole of the substance to be titrated, the nitrogen values were recalculated to molarities on the basis of 145 and 141 nitrogen atoms per molecule for cytochrome c and its protein moiety respectively. Thus the former contained 9.83×10^{-7} and the latter 10.12×10^{-7} moles per ml. 5.00 ml of the solutions to be titrated (cytochrome, free protein and water in the case of the blank curve) + 5.0 ml glycerol a. g. + 0.05 ml octanol were acidified with 0.28 ml 1.011 *M* HCl. An iron rod, sealed in glass, was put in the vessel for the magnetic stirring. To protect the solution from CO_2 the vessel was closed with a rubber membrane, which also served to keep in position the in- and outlet tubes for the CO_2-free nitrogen stream, the glass electrode, the agar bridge with saturated KCl, and the microburette with sodium hydroxide (2.530 *M*). The tip of the burette and the inlet tube for the nitrogen stream dipped a few mm below the surface of the liquid. The electrode and the potentiometer (Cambridge Instruments) were checked with standard phtalate (pH 3.97) and borate (pH 9.14 at 25° C) buffers before and after each titration. The temperature was kept constant by means of a water thermostat. The results of the titrations are discussed on page 384.

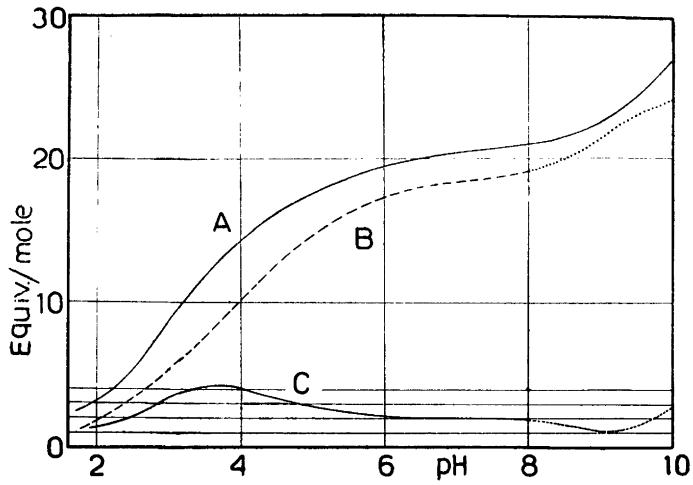

Fig. 3. Titration curves of cytochrome c and its protein moiety in glycerol-water 1 : 1. The positions of the two curves in relation to each other have been given so they should differ by two equivalents at pH 7, where all groups, common to the two substances, are titrated and in addition to them the two ferri porphyrin carboxyl groups in cytochrome c. The absolute positions along the ordinate are arbitrarily chosen. The same applies to the difference curve, and thus only differences in equivalents per mole between two pH values can be obtained. Curve A: Cytochrome c. Curve B: Protein moiety. Curve C: Difference between A and B.

The above-mentioned methods for studies of the heme-linked groups give only indirect information about the nature of these groups. For that reason it was desirable to employ some method, which could give a more direct answer. 1,2,4-fluorodinitrobenzene (FDNB) reacts with the imidazole group of histidine to give DNB-histidine [5]. The amino group will also combine with FDNB, but if this group has been protected by a peptide bond during the exposure to FDNB, histidine, subsequently liberated by acid hydrolysis, contains only two polar groups. Since the amino acid decarboxylases can not attack amino acids with only two polar groups, histidine with DNB bound to the imidazole ring will not be decarboxylated. On the assumption that their linkages to the iron atom would prevent two of the imidazole groups from reacting with FDNB [6], the following experiment was made (Table 1).

Histidine decarboxylase [7] and FDNB [8] were prepared according to given descriptions. The latter substance could not be distilled completely free from the yellow colour, although it was definitely lighter than the commercial preparation. The cytochrome c for this experiment was purified in the same way as the preparation used for the titrations, and had an iron content of 0.426 %. The amount of histidine residues, able to react with histidine decarboxylase, was determined in two hydrolysates of cytochrome with and without pretreatment with FDNB. In the first case 7.44×10^{-6} moles in 5 ml water + 6.25 ml conc. HCl were refluxed on a sandbath for 24 h, cooled, filtered, and evaporated to dryness several times with addition of water. The residue was dissolved in 0.2 *M* acetate buffer pH 4.5 and pH adjusted to 4.5 with 1 *M* NaOH. The final volume was 6.0 ml. Aliquots of this were taken for manometrical determinations of CO_2 evolved due to

Table 1. Determination of free histidine in hydrolysates by means of enzymatic decarboxylation. Temperature + 30° C. Side bulb: 40 mg (dry weight) bacterial cells in 0,50 ml. 0.2 M acetate buffer pH 4.5. Volume in main vessel made up to 3.15 ml with acetate buffer. Histidine solution 8.30 × 10^{-6} moles per ml. Cy hydr. = hydrolysate of untreated cytochrome c. DNB-cy hydr. = Hydrolysate of FDNB-treated cytochrome c.

Material	μl CO_2		
	Found	Calc.	Found %
Blank	<2	—	—
0.75 ml histidine	132	155	85
1.50 » »	274	310	88
		Average recovery 86.5	
		Moles of histidine per mole of cytochrome	
1.00 ml cy hydr.	81	2.63	
2.00 » » »	154	2.50	
1.00 » DNB-cy hydr.	29	0.47	
2.00 » » »	58	0.47	

the action of the enzyme. In the second case 1.95 × 10^{-5} moles of cytochrome c in 5 ml 10 % $NaHCO_3$ were shaken with 1 ml FDNB in 9 ml ethanol for two hours at room temperature [9]. The protein precipitate was washed four times with ethanol and twice with ether. It was then dried and hydrolyzed as above. The final volume of the solution in buffer after the correction of pH was 7.8 ml.

DISCUSSION

The sulphur containing amino acids. Cytochrome c contains six atoms of sulphur per molecule [1]. Two of them derive from methionine, and another two were found in the cystine fraction [10]. The latter two were considered as identical with the thio-ether forming cysteine residues. However, native cytochrome c gives a polarographical wave indicating the formation of a thiol group [11]. The parent group of that thiol can not be methionine [11] or the thio-ether group [12]. Thus it is very likely that cytochrome c contains one cystine in addition to the porphyrin bound cysteine residues. The nitroprusside reaction for free sulphhydryl groups is negative for cytochrome c [1]. In experiments with free amino acids we found that after treatment under the experimental conditions employed for the splitting of the cytochrome, cystine but not methionine gives a positive nitroprusside reaction. Thus the protein moiety of cytochrome c should contain two thiol groups + one disulphide group, or, in

the presence of silver ions, four mercaptide bound silver atoms per 141 nitrogen atoms. Peters and Wakelin [13] found that methionine gave a positive nitroprusside reaction after warming only at slightly alkaline reaction. This means that it might be possible to determine cystine on the basis of the silver content of the protein after digestion at faintly acid reaction and the sum of cystine and methionine after digestion at faintly alkaline reaction. The amount of sulphur found in cystine and methionine should for most proteins be equal to the total sulphur content of the protein. The hydrolysis of the protein with strong acid with the losses of cystine could thus be avoided.

Titration curves. In agreement with the described theory the differences in the titration curves of cytochrome c and its protein moiety can be interpreted in the following way: In intact cytochrome c the group of pK 0.42 is outside the titrable range because the lowest pH at which cytochrome c is stable is 1.7 [14]. From pH 1.7 to pH 4 the intact cytochrome c consumes three equivalents more than the free protein does. These three groups correspond to the two carboxyl groups of the ferri porphyrin + the imidazole group of pK 2.50. In the free protein the ferri porphyrin carboxyl groups are absent, and the imidazole groups are titrated closer to their normal pK value. Hence the free protein should consume two equivalents of base per mole more than the intact cytochrome does between pH 4 and neutrality. The dissociation constant in the free protein at about pH 8 may be attributed to an amino group in the neighbourhood of the porphyrin carboxyl groups as was done in the case of the ferrihemoglobin — globin titrations [15].

A few comments have to be made to this interpretation.

1. Cytochrome c itself is stable within the pH region covered in the titrations [14]. The best evidence for the stability of the protein moiety would of course be to recombine it with the prosthetic group and demonstrate the biological activity of the product. It is, however, very unlikely that a recombination to a substance, possessing the thio-ether bonds, could take place under the conditions usually employed for the recombination of ferri porphyrins with enzyme protein components (aqueous solution of pH about 7). A mixture of the protein moiety and the ferri porphyrin under such conditions gives upon reduction a spectrum, very similar to that of ferrocytochrome c; however, since acid acetone splits off the prosthetic group, no recombination to the structure of cytochrome c can have occurred. For that reason it has not been possible to test the stability of the free protein. The whole discussion has therefore to be based on the assumption that the protein part can stand the treatment without changes in its acid-base combining capacity in addition to those which are caused by the removal of the prosthetic group. The amide nitrogen was unchanged by the silver sulphate — acid acetone treatment.

2. There is evidence in support of the assumption that the porphyrin carboxyl groups in cytochrome c are free to be titrated. Hematoporphyrin can not be prepared from ferri protoporphyrin chloride by means of hydrogen bromide in glacial acetic acid if the porphyrin carboxyl groups are linked to amino acids by peptide bonds [16], but cytochrome c gives readily hematoporphyrin by that treatment [17]. Moreover it seems unlikely that the procedure for the splitting of cytochrome c, employed in this paper, could break a peptide bond. The interpretation of the titration curves presupposed that the ferri porphyrin carboxyl groups were titrated at about pH 3, far below their normal pK value. p$K_{25°}$ for propionic acid in water is 4.87. The di-sodium salt of ferri protoporphyrin hydroxide ("hematin") consumed upon its titration with hydrochloric acid simultaneously two equivalents at about pH 6.5 [18]. No formation of the mono-salt could be seen. In porphyrin c [19] two of the four carboxyl groups were titrated with pK 5.7. In both examples, however, the carboxyl groups were uninfluenced by positive groups in the vicinity. A positively charged—NH_3^+ group in the neighbourhood would increase the tendency of the carboxyl group to split off a proton. In fact, the low pK value for the carboxyl groups indicates that they are influenced, *e. g.* by salt linkages, by positive groups of the protein.

3. If two protein amino groups are sterically close to the ferri porphyrin carboxyl groups, the ionization of pK about 8 should correspond to two equivalents instead of only one. However, one of these groups may of course be strongly basic and not appearing within the examined pH range. It may also be that the flocculation of the protein, beginning just there, could mask one amino group.

4. It should be mentioned that some preparations of cytochrome c consumed more than two equivalents between pH 5.5 and 8.5. The preparation, the titration curve of which is reproduced in Fig. 3, thus consumed 2.9 equivalents. It may be that this specimen was not pure. Since this investigation was finished, cytochrome c with an iron content of 0.47 % has been obtained [20, 21]. The nature of the fraction, removed by these investigators, as well as its relation to the cytochrome molecule, is as yet unknown.

As a whole the results from the titration experiments can be interpreted in agreement with the theory of histidine residues as heme-linked groups, but they do not exclude the possibility that other groups are heme-linked. This possibility will therefore be briefly discussed.

Tsou [22] digested cytochrome c with pepsin at pH 1.5, and isolated a peptide of the average molecular weight 2 500, calculated on the basis of its iron content. This peptide, which was autoxidizable, contained the prosthetic group plus some amino acids. It lacked the absorption band at 280 mμ, deriving

from tyrosine and tryptophane, but it contained histidine according to its paper chromatogram. Thus tyrosine can be excluded as mediator of the protein-iron linkage. Moreover, an ionization due to the hydroxyl group of tyrosine should appear at much higher alkalinity than between pH 4 and neutrality.

The average value of 2.57 histidine residues per mole of cytochrome c was found by the determination with histidine decarboxylase. If this value is corrected for the average recovery of 86.5 %, observed with pure histidine, it will increase to 2.97, in good agreement with the value from amino acid analyses. After the same correction the value for the DNB-cytochrome becomes 0.54. This is considerably lower than the expected value of 2, which was calculated from the assumption that both heme-linked groups were histidine residues. However, cytochrome c is largely denatured by alcohol. Since it was possible that the denaturation could imply a re-arrangement around the iron atom, the following experiment was made. A 2×10^{-5} M solution of cytochrome c in 10 % $NaHCO_3$ was divided into two equal parts. One part was treated with alcohol but without FDNB and dried as described above. It was then re-dissolved to its original volume with water. Both solutions were then reduced with the same quantity of dithionite and aerated. The alcohol treated cytochrome c was rapidly reoxidized, while the other remained reduced. Thus the alcohol treatment itself makes cytochrome c autoxidizable. This means that oxygen somehow can reach the iron, and it might thus also be possible for FDNB to react with at least one of the heme-linked groups. The experiment with FDNB gives therefore only qualitative indication that one of these groups is an imidazole. In the case of hemoglobin [6] the hemin did not protect the histidine residues from reacting with FDNB, and free hemin could be detected in the solution after the exposure to FDNB-alcohol. The better results with cytochrome c can therefore be attributed to the firmer heme-protein bonds.

Thus from experiments, presented earlier [1, 22] and in this paper it can be considered as established that at least one histidine residue is linked to the iron. From Fig. 3 it is obvious that also the other group must have its pK value between pH 4 and neutrality. For this reason a guanidino group can be excluded as heme-linked. Possible groups — in addition to the imidazole group — could be the second carboxyl group of aspartic or glutamic acid. In the free amino acids these groups have the pK values 3.65 and 4.25 respectively in water. In peptides and in solvents of lower dielectricity the values are slightly higher [23]. It is thus possible that one of these acids could constitute the other heme-linked group. It is likely that two different pK values should be found for the groups dissociating between pH 4 and 6.5 if they were one

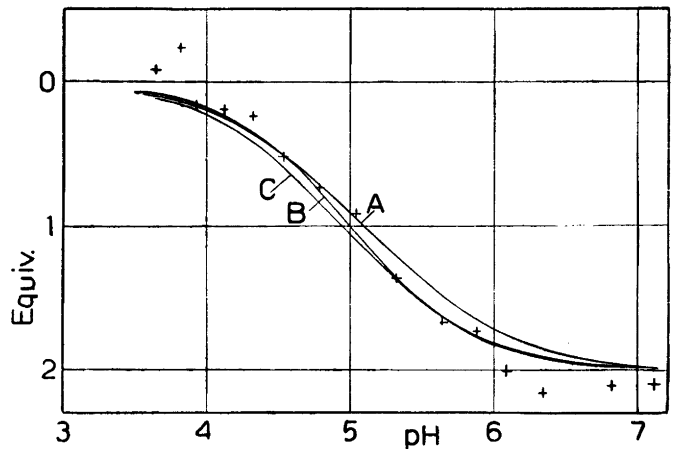

Fig. 4. The difference curve between pH 4 and 7 (excerpt from Fig. 3). + experimentally determined differences. The curves have been drawn on the assumptions that two equivalents are titrated between pH 4 and 7 with the pK values for curve A 4.70 and 5.50, curve B 4.90 and 4.90 and curve C 4.70 and 5.20.

imidazole and one carboxyl group. In Fig. 4 the difference in base-binding capacity between cytochrome c and its protein moiety within this region are plotted against pH. The curves are calculated for the dissociations of two groups of certain pK values. As can be seen the accuracy of the experiments does not permit a definite conclusion about which curve would fit best, though it seems to be the one with two equal pK values.

SUMMARY

1. Cytochrome c and its protein moiety have been titrated in glycerol-water between pH 1.7 and 8. Between pH 1.7 and 4 cytochrome c consumed three equivalents per mole more than its protein moiety. From pH 4 to pH 6 the latter took up two equivalents more, and between pH 8 and 9 another equivalent. The results are compatible with the theory that histidine residues constitute the heme-linked groups, but the possibility that one carboxyl and one imidazole group are linked to the iron can not be excluded from these experiments. When cytochrome c was treated with flurodinitrobenzene, hydrolyzed, and the amount of free histidine determined by means of histidine decarboxylase 0.54 moles of histidine, free from dinitrobenzene, were found per mole of cytochrome c. This result indicates that at least one histidine residue is linked to the iron atom.

2. A discussion of data, available in the literature, reveals that cytochrome probably contains two methionine, two cystine, and two cysteine (thio-ether bound) sulphur atoms. From results, obtained by treatment of the free, sulphur containing amino acids in the same way as was done with cytochrome c, it was to be expected that the protein moiety should contain four silver atoms per mole. The value actually found was 3.5—3.6. It is suggested that this observation could give a new method for the determination of the sulphur containing amino acids.

During a visit to England, made possible by a grant from *Statens Medicinska Forskningsråd*, the author had the opportunity to study the determination of histidine by the decarboxylase method. He wants to express his thanks to dr E. F. Gale and dr J. Tosic at the Unit of Microbiology, Cambridge, for advice and discussions. The author's thanks are also given to prof. H. Theorell for very valuable discussions, and to miss Inger Agerberg for technical assistance.

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