Chemical Studies on the Homogeneity of the Structural Protein from Mitochondria*

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ABSTRACT: The protein fraction from mitochondria, which has been designated structural protein and which has been considered to function in ordering and stabilizing mitochondrial structure, has been investigated.

A single *N*-acetylated amino acid has been found in the preparation. Analysis of tryptic hydrolysates of this fraction are consistent with its existence as a single protein species. The ability of structural protein to form complexes by reaction with heme protein is lost upon aging of the sample with a parallel decrease in sulfhydryl titer and increase in weight-average molecular weight. This ability can be regained by disulfide bond reduction.

The mitochondrion has been demonstrated to contain a protein fraction termed structural protein, which accounts for 40–50% of the total mitochondrial protein (Green *et al.*, 1961). This fraction has been ascribed the role of a membrane protein which functions both in stabilizing mitochondrial structure in the characteristic double membrane organization and in interacting with the various cytochromes and the mitochondrial lipids to form an organized enzyme complex (Criddle *et al.* 1962). The structural protein from beef heart mitochondria has been shown to be a protein of 22,500 g/mole which appears electrophoretically and centrifugally homogeneous. Evidence for a single C-terminal amino acid in this preparation was demonstrated using the carboxypeptidase method. However, no amino terminal amino acid could be detected. Structural protein is only soluble in aqueous solution at neutral pH values. It can, however, be solubilized by either going to extremes of pH or by use of detergents such as sodium dodecyl sulfate (SDS).[1] Solubilization can also be effected by interaction with purified cytochrome components. In this case, a mutual solubilization occurs through a specific interaction between two aggregating proteins with the result that both are solubilized in the process. Stoichiometric complexes of structural protein and the cytochromes *b*, c_1, and cytochrome oxidase have been demonstrated.

A study has been undertaken to investigate the chemical homogeneity of the structural protein and evidence is presented to indicate that this large fraction of protein is in fact a single protein species. Factors affecting the ability of the structural protein to form complexes with the cytochromes have also been studied.

Methods and Materials

Structural Protein Isolation. Structural protein was isolated from beef heart mitochondria by the method of Richardson *et al.* (1964). This procedure, which is a modification of the original procedure of Green *et al.* (1961), involves solubilization with cholate and deoxycholate followed by ammonium sulfate precipitation and removal of the bile acids and lipids with acetone–water washes.

Peptide Mapping. Structural protein was first oxidized with performic acid using the procedure of Hirs (1956) to break all disulfide bonds. Performic acid oxidation was effective in rendering the protein susceptible to enzymatic hydrolysis but has the disadvantage of destroying tryptophan. Proteolysis was carried out using Sigma two times crystallized trypsin which had been pretreated with PTCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone) to inhibit any chymotryptic activity (Schoellman and Shaw, 1963). Trypsin was added to a suspension of structural protein (30 mg/ml) maintained at pH 8.3–8.5, at an enzyme–protein ratio of 1:60, and allowed to incubate at 37° for 12 hr. At the end of this time 92–94% of the structural protein had been solubilized as peptides. The residual protein was removed by centrifugation and discarded. The peptides were lyophilized and taken up in 0.001 M potassium phosphate buffer at pH 7.5.

Two-dimensional peptide maps of structural protein were prepared using the procedure of Katz *et al.* (1959). Electrophoresis of the sample was run in pH 6.4 buffer of pyridine–acetic acid–water (25:1:225), followed by chromatography at right angles using freshly prepared butanol–acetic acid–water (4:1:5) as a solvent. Peptides were determined by spraying with a 0.2% solution of ninhydrin in acetone and drying at 80°. The Sakaguchi

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[1] Abbreviations used: SDS, sodium dodecyl sulfate; PTCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

method (Block *et al.*, 1953) was used for determining the positions of arginine-containing peptides on the chromatograms. Two-dimensional electrophoresis was also employed using pyridine buffer in one dimension and pH 3.9 citrate buffer in the other.

Determination of Acetylated Amino-Terminal Amino Acid. A suspension of structural protein was treated with pronase (obtained from California Biochemical Corp.) at a concentration of 0.2 mg/ml and a weight ratio of protein to enzyme of about 100:1. The mixture was incubated with shaking at 37° for 12 hr in pH 8.0, 0.05 M phosphate buffer. The protein suspension was solubilized as the pronase digested the sample to a mixture of amino acids and small peptides. Pronase does not remove the acetate group from *N*-acetyl amino acids (Nomoto *et al.*, 1960). The hydrolysate was then acidified to pH 3.1 and placed on a 20 × 2 cm Dowex-50 column which was equilibrated with pH 3.1, 0.05 M sodium acetate buffer. Fractions (5 ml) were collected for the first 100 ml of eluate and were analyzed for amino acids or substituted amino acids using acid hydrolysis in 6 N HCl followed by ninhydrin determination.

A second procedure used for removing amino substituted amino acids or peptides from the pronase digest was to acidify the digest to pH 1.0 with HCl and then extract four times with four volumes of ether. The ether extracts were then evaporated to dryness under a stream of air and the residue dissolved and analyzed for amino acids and substituted amino acids as above.

Samples from each of the above preparations were hydrolyzed in 6 N HCl at 110° for 4, 8, and 12 hr and the hydrolysates were chromatographed along with the unhydrolyzed samples and with ^{14}C-*N*-acetylserine on Whatman No. 3 paper in the solvent systems: (1) butanol–acetic acid–water (40:10:50) and (2) *t*-amyl alcohol saturated with water. The papers were developed with ninhydrin spray. Chromatograms of the unhydrolyzed sample were cut out and eluted at the position corresponding to ^{14}C-*N*-acetylserine. The eluate was then hydrolyzed with HCl and rechromatogramed to determine the presence and identification of amino acids.

Chromatography of *N*-acetylserine and the substituted amino acids obtained from the pronase digest of structural protein was also carried out using the procedure of Whitehead (1958) for the detection of acyl amino acids directly on paper.

N-Acetyl groups were determined colorimetrically by the method of Ludowieg and Dorfman (1960). Deacetylation was carried out in HCl–methanol. Methyl acetate is formed and determined as a hydroxamic–ferric complex.

Preparation of ^{14}C-N-Acetylserine. Labeled *N*-acetylserine was prepared by adding 0.01 ml of C^{14}-labeled acetic anhydride (specific activity = 4.5 μcuries/μmole) to a 0.01 M serine solution at pH 7.0 and allowing the reaction to proceed at 0° for 2 hr. The reaction mixture was then acidified to pH 2.0 and extracted 10 times with five volumes of ether. The ether extract was then evaporated to dryness to remove acetate, and

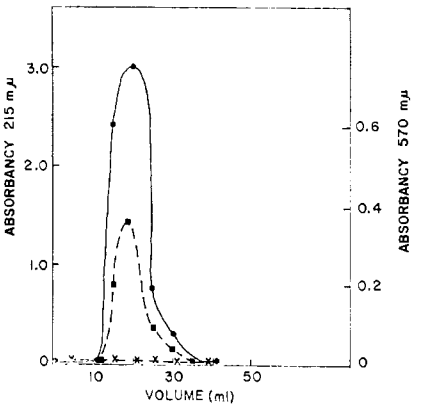

FIGURE 1: Elution of pronase digest of structural protein from Dowex-50 column. Fractions (5 ml) were collected and followed by measuring optical density at 215 mμ (●——●) and at 570 mμ using the ninhydrin reaction (–×–×–). Aliquots of each fraction were removed, hydrolyzed with HCl, and again subjected to the ninhydrin reaction (–■–■–■–).

dissolved in water. Quantitative estimation of amino acids was carried out by Rosen's (1957) method.

Sulfhydryl Determination. Structural protein was solubilized in pH 8.0, 0.05 M sodium phosphate buffer containing 0.1% sodium dodecyl sulfate. The sulfhydryl content of various dilutions of this solution was determined colorimetrically using the reagent DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) according to the method of Ellman (1959). Solubilization of the protein in 2% sodium dodecyl sulfate or in 6 M urea had no effect on the amount of SH determined.

Free sulfhydryl groups on the structural protein were blocked by reaction with excess iodoacetate or iodoacetamide (Matheson Coleman and Bell) or with *p*-mercuribenzoate (Sigma) at pH 9.0 in Tris buffer.

Sedimentation and molecular weight measurements were carried out in a Spinco Model E ultracentrifuge. Molecular weight measurements were made by Ehrenberg's (1957) method of approach to sedimentation equilibrium or by the short column equilibrium method of Van Holde and Baldwin (1958).

The method of fluorescence quenching (Udenfriend, 1962; Edwards and Criddle, 1966) was used to follow the binding of structural protein to heme proteins and to determine the fraction of the protein capable of forming complexes.

Results

N-Terminal Amino Acid Analysis. No N-terminal amino acid was found for structural protein preparations using either the fluorodinitrobenzene or the phenyl isothiocyanate method. Pronase hydrolysis followed either by separation of the acid solution using chromatography on Dowex-50 resin or by ether extraction yielded ninhydrin-negative products which behaved identically in all subsequent analyses. Elution

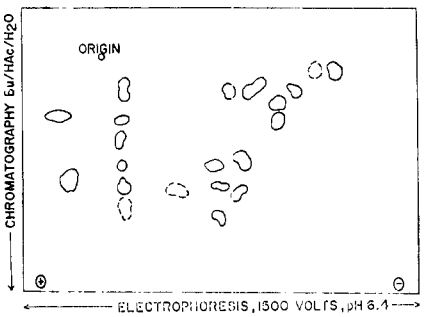

FIGURE 2: A representative tracing of a "fingerprint" of structural protein.

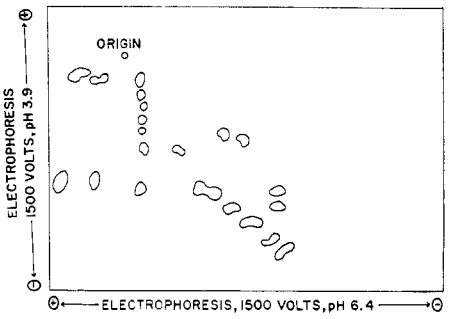

FIGURE 3: Two-dimensional electrophoresis of tryptic digest of structural protein.

of the pronase hydrolysate from Dowex-50 was followed at 215 mμ and by ninhydrin reaction with the eluate and the HCl hydrolyzed eluate (Figure 1). Acid hydrolysis of the eluate or of the ether extracts from the pronase digest yielded a ninhydrin-positive compound which chromatographed on paper as serine in two different solvent systems. A second faint spot was usually observed at high concentrations which chromatographed as alanine. Based on a series of dilutions, this appears to represent no more than 5% of the concentration of the serine.

Chromatography of the pronase digestion product using the solvent system and direct staining procedure for *N*-acyl amino acids of Whitehead also correlated the movement of the digestion product with that of *N*-acetylserine. Acetyl determination on the protein demonstrated on multiple analysis that 1 mole of acetyl group could be found per 22,000–26,000 g of protein (Table I).

TABLE I: Determination of Acetyl Groups on Mitochondrial Structural Protein.

Sample Size (mg)	Acetyl/ Sample (μmoles)	Wt of Protein/ Mole Acetyl Group (g)
42	1.65	25,200
25	1.12	22,200
21	0.89	23,600

Peptide Mapping. On the basis of amino acid analysis and a molecular weight of 22,500 g/mole structural protein contains 20–21 lysine plus arginine residues per molecule (Criddle *et al.*, 1962). On complete digestion with trypsin one should expect to obtain 21–22 peptides if the protein is a homogeneous species and if there are no repeating subunits. If the preparation is heterogeneous, then one should expect an increased number of peptide fragments. With a very heterogeneous preparation, large numbers of peptides would be observed, many of which could however be very faint. Figure 2 shows a representative pattern of a "fingerprint" of structural protein. Twenty-one spots are repeatedly observed on the ninhydrin-developed chromatograms. This agrees well with the predicted number of peptides assuming a single protein species in the preparation of structural protein. Two-dimensional electrophoresis of the tryptic digest also indicated the presence of 21 strong peptides (Figure 3).

The possibility exists that a heterogeneous system of proteins could give rise to only the 22 strongly detected peptides observed here due to lack of resolution, poor digestion by the proteolytic enzyme, or weakly staining peptides which may be present in only small amounts. This possibility was investigated using the Sakaguchi reagent to stain for arginine-containing peptides. The chromatogram of Figure 4 demonstrates eight positively staining peptides with this reagent, which again agrees well with the amino acid analysis which indicates 7.9 moles of arginine per 22,500 g of structural protein.

Relation between Sulfhydryl Content and Binding of Heme Proteins. The sulfhydryl content of freshly prepared samples of structural protein was determined using the Ellman Reagent to be 1 mole of SH per 20,800 ± 2000 g of protein. This value was not changed by carrying out the determination of either high SDS concentrations (2%) or in 6 M urea. There was a definite loss in SH titer, however, upon aging of the structural protein preparation. The sulfhydryl content of stock solutions of SP as normally prepared decreased only slowly on standing over a period of several days when stored at 5°. This process could be accelerated markedly by maintaining the sample at room temperature at pH 8.5 and in the presence of either high SDS concentrations or air bubbling through the solution. Results of standing at room temperature at pH 8.5 in the presence of 2% SDS are shown in Table II.

The loss in sulfhydryl content is paralleled by an increase in the weight-average molecular weight of the

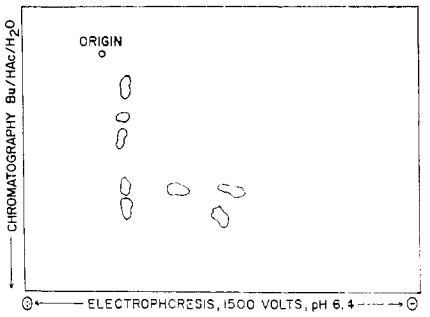

FIGURE 4: "Fingerprint" of tryptic digest of structural protein. The spots were developed using the Sakaguchi reaction.

TABLE II: The Effect of Aging upon the SH Titer and the Molecular Weight of Structural Protein.

Time[a] (hr)	Moles of SH/Mole of Protein[b]	Wt-Av Mol Wt (g/mole)
0	1.06	22,500[c]
20	0.30	39,000[d]
41	0.22	—
63	0.16	43,000[d]

[a] Zero time taken as time at which freshly prepared sample of structural protein is solubilized in 2% solution of SDS in pH 8.5, 0.05 M sodium phosphate buffer. [b] Based on molecular weight of 22,500 g/mole. [c] Determined by the method of Van Holde and Baldwin (1958) in pH 9.0, 0.05 M Tris buffer at a protein:SDS ratio of 10:1 (w/w). Correction for bound detergent was made. [d] Molecular weights were determined by the method of Ehrenberg (1957) after dialysis of solution against pH 8.5, 0.05 M sodium phosphate buffer for 12 hr to remove any dialyzable detergent. No attempt was made to correct the above values for bound detergent.

sample (Table II) and appears to explain the earlier observations of the formation of a species having the molecular weight of a structural protein dimer (Criddle *et al.*, 1962). The weight-average molecular weight of the structural protein preparation is found to approach 44,000 g/mole after prolonged standing. The loss in sulfhydryl content also parallels a decrease in the ability of the structural protein to interact with the cytochromes of the mitochondrion and with myoglobin. Using the method of fluorescence quenching to study the complex formation between the structural protein and heme proteins, it has been possible to determine the thermodynamics of the interaction and also to determine the fraction of the structural protein preparation which is involved in the binding process (Edwards and Criddle, 1966). The fraction of the protein capable of binding has been shown to decrease with the

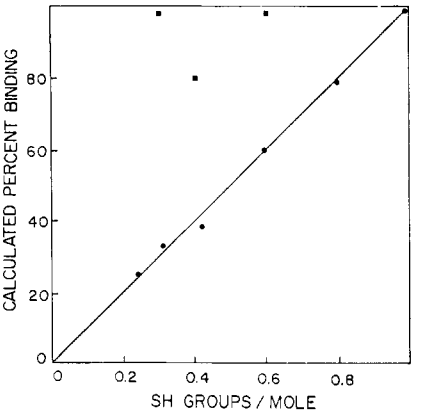

FIGURE 5: Relation between titratable sulfhydryls of structural protein and cytochrome *c* binding capacity. The points are the experimentally observed relationship between titratable SH and the binding capacity of structural protein preparations. The straight line indicates a theoretical 1:1 correlation between SH titer and binding. The boxes -■- indicate the measured binding of corresponding titrated samples after treatment with dithioerythritol.

aging of the sample while the dissociation constant for the remaining material is unchanged. Figure 5 shows that the loss of sulfhydryl titer directly parallels the decrease in the fraction of the structural protein molecules which are capable of binding myoglobin. A similar curve is obtained for binding of cytochrome *c* to the aged preparation. Upon addition of 0.001 M dithioerythritol to the structural protein and a 5 min incubation to allow protein disulfide reduction, an increase in binding capacity of the aged samples can be noted to the level that 80–100% of the structural protein again participates in the binding (see Figure 5).

While loss of sulfhydryl titer due to aging and disulfide bond formation prevents the binding of heme proteins to structural protein, the sulfhydryl itself is not directly involved in the complex formation. Incubation of the structural protein with iodoacetate, iodoacetamide, or *p*-mercuribenzoate to block the free SH groups had no effect on its ability to specifically interact with the cytochromes.

Discussion

The data presented in this paper are consistent with the belief that the structural protein isolated from beef heart mitochondria is a single protein species and that each molecule can participate in the binding of heme proteins. Earlier evidence had indicated the presence of a preparation homogeneous to physical measurements but could not indicate chemical homogeneity of this large protein fraction which constitutes approximately 40% of the mitochondrial protein.

The finding of a single *N*-acetylserine molecule per structural protein molecule of 22,500 mol wt suggests

that the reason for failure in earlier attempts at locating an N-terminal amino acid was due to existence of the acylated derivative in the terminal position. If this is the case, the identification of a single N-terminal amino acid is strong evidence for the homogeneity of the preparation. It is interesting to note that *N*-acetylserine has also been identified as the terminal amino acid in tobacco mosaic virus coat protein and that the N-terminal amino acid of structural proteins from many sources have been found to be *N*-acetylated (Narita, 1958).

The agreement between amino acid analysis and number of peptides observed in the "fingerprinting" technique could be fortuitous. Peptide mapping of the ribosomal coat protein, for example, has yielded only a small number of peptides in spite of the fact that several protein species have been demonstrated to be present in the preparation. For one to obtain the correct number of peptides for agreement with amino acid analyses from a mixture of proteins and then also to obtain agreement with the number of arginine-containing peptides from the same mixture is less likely, however. These data are, therefore, again indicative of a single species of structural protein.

The third line of evidence for homogeneity of structural protein preparation is indicated by the 1:1 correlation between the fraction of structural protein binding heme proteins and the sulfhydryl content of the preparation. It has been demonstrated that all of the structural protein molecules can bind cytochromes. Loss of SH titer, presumably due to oxidation giving rise to disulfide bond formation between SP molecules, results in dimer formation and prevents complexing with the heme proteins. For each mole of SH disappearing, a mole of binding capacity is lost without detectable change in the binding energy of the remaining material. Regeneration of sulfhydryls using dithioerythritol restores the ability of the molecule to bind cytochromes. Thus, a binding site must exist on each of the structural protein molecules which becomes less accessible upon dimerization but which can be reversibly recovered. Evidence for the homogeneity of the binding sites present on the structural protein is presented in detail elsewhere (Edwards and Criddle, 1966).

Acknowledgment

We wish to acknowledge the technical help of Mrs. Linn Park in protein preparations and in performing some of the fingerprint analyses.

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