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To the Graduate Council:

I am submitting herewith a dissertation written by Beth Feland entitled "Isolation and Characterization of a High Energy Iron-Sulfur Protein From Mammalian Cells." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutrition.

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Major Professor

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ISOLATION AND CHARACTERIZATION OF A HIGH ENERGY IRON-SULFUR PROTEIN FROM MAMMALIAN CELLS

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Beth Feland

August 1973

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ABSTRACT

A sulfur-containing protein (S-protein) was proposed to be an intermediate in the sulfation of cellular constituents containing ester sulfate after it was observed that S-protein isolated from the particulate fraction of the cell was unaffected by avitaminosis E, the stress of malathion intoxication or the level of inorganic sulfate in the diet. The present investigation was undertaken in an attempt to identify the cellular role of the S-protein in sulfate utilization.

The role of the S-protein as a possible intermediate in the sulfation of ester sulfates was determined using both a natural sulfate acceptor, mucopolysaccharide, and an artificial sulfate acceptor, p-nitrophenol. The S-protein appeared to possess the energy required to raise inorganic sulfate to the level of ester sulfate when mucopolysaccharide was the acceptor, but not when the artificial acceptor, p-nitrophenol, was used.

Since the S-protein appeared to possess a high energy configuration, and in view of its particulate origin, further experiments were designed to determine the relationship of the high energy configuration to the oxidative reactions of the cell. Since other investigators had previously suggested that an Fe-S protein is involved in sulfhydryl-disulfide oxidoreductions in the energy conservation at Site I, the modified sulfhydryl-disulfide exchange reaction between the S-protein and 2-mercaptoethanol led to the assumption that the S-protein might be involved in energy conservation at Site I.

The evidence that the S-protein (Fe-S protein) was involved in energy conservation was strengthened by feeding rats 2,4 dinitrophenol, an uncoupler of oxidative phosphorylation, prior to isolation of Fe-S protein. Results of this experiment and an experiment determining the uptake of a test dose of $^{59}Fe^{++}$ and $^{35}SO_4^{=}$ suggested that rats increased the synthesis of Fe-S protein to compensate for the decreased energy conservation that resulted from the injection of the uncoupler of oxidative phosphorylation.

The relationship between the Fe-S protein and the formation of ATP was then investigated. When the Fe-S protein was incubated with inorganic phosphate and ADP there was an increase in the disappearance of phosphate which paralleled the amount of Fe-S protein in the incubation mixture. The actual esterification of inorganic phosphate with ADP was demonstrated using $^{32}PO_4^{\equiv}$ in the incubation medium and isolating pure ATP containing ^{32}P. Therefore, it is concluded that the Fe-S protein functioned to trap a portion of the energy of the respiratory chain by oxidatively binding inorganic sulfate, which it can then exchange for inorganic phosphate. The high energy phosphate could then be transferred to ADP to generate ATP.

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CHAPTER I

INTRODUCTION

Fractionation of a ^{35}S-cellular lipoprotein preparation from rat liver cells gives a lipid, a mucopolysaccharide, a nucleotide, and an unidentified protein fraction (S-protein), all of which contain ^{35}S-activity (1). Fulton and Smith (1) investigated the effect of avitaminosis E upon the sulfation of cellular lipoprotein and its fractions by feeding diets low in inorganic sulfate and deficient in vitamin E or supplemented with α tocopheryl acetate. They found that avitaminosis E resulted in a decreased ^{35}S-sulfate incorporation into cellular lipoprotein, and its lipid and mucopolysaccharide fractions. However, there was no effect on the incorporation of ^{35}S-sulfate into the S-protein. Determination of the incorporation of ^{35}S-sulfate into the S-protein with time demonstrated that it became maximally labeled sooner and lost its label earlier than any of the other cellular lipoprotein fractions. Based on the rate of turnover of ^{35}S-sulfate, S-protein appeared to have a precursor relationship to the rest of the fractions which suggested the possibility that the S-protein might serve as an intermediate in the sulfation of other ester sulfates in the cell.

In later experiments Disney (2) found that the stress of malathion intoxication resulted in an increase in the uptake of ^{35}S-sulfate into the sulfolipid component of the cellular lipoprotein, but did not affect the incorporation of ^{35}S-sulfate into cellular lipoprotein nor its mucopolysaccharide or S-protein fractions.

Since neither avitaminosis E nor the stress of malathion intoxication affected the uptake of ^{35}S-sulfate by the S-protein, it appeared that the unidentified protein fraction of cellular lipoprotein was a high priority protein with respect to sulfate. Therefore, this investigation was undertaken in an attempt to identify the possible role of the S-protein in sulfate utilization.

CHAPTER II

REVIEW OF THE LITERATURE

Mitochondria contain a series of multienzyme pathways closely associated with, or an integral part of, the inner mitochondrial membrane. Their physiological function is to catalyze the oxidation of intermediary metabolites by molecular oxygen in such a way that the energy released by these oxidation-reduction reactions may be conserved in a form that can be utilized by the cell for energy requiring reactions (3). The process of energy conservation in the mitochondria requires the presence of several systems: 1) a chain for the transfer of electrons, 2) a system for generating the electron donors for the chain, and 3) a system for synthesizing adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (4).

The respiratory chain is a structural group of proteins containing oxidation-reduction groups which implement the stepwise (but not continuous) transfer of electrons from electron donors, such as reduced nicotinamide adenine dinucleotide (NADH), and succinate, to molecular oxygen. The group of proteins of the respiratory chain can be subdivided into four units. These units have been referred to as Complexes I, II, III and IV as shown in Figure 1. In the oxidation of NADH, Complexes I, III and IV follow one another successively while in the oxidation of succinate, Complexes II, III and IV provide the pathway for the flow of electrons (4,5).

As a result of the passage of electrons through Complexes I, III and IV, an energized state of the complex is generated in which the free

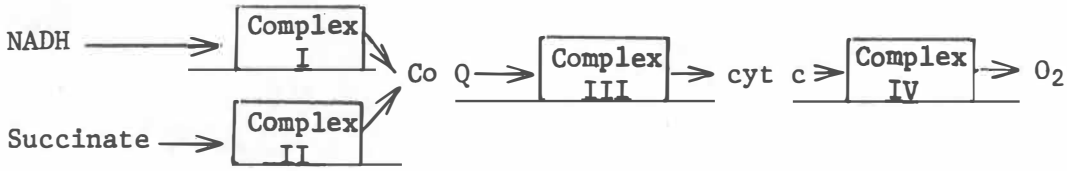

Figure 1. Schematic representation of the arrangement of the complexes in the mitochondrial respiratory chain.

energy released by the oxidoreductions is conserved and trapped by the formation of ATP (4). The exact sites in these complexes where oxidative phosphorylation occurs have not been identified with certainty. The three sites of oxidative phosphorylation are referred to as Sites I, II and III. The nature of the electron transfer is different at each of the three sites and the actual mechanism for coupling the energy released during the oxidation of the electrons to the synthesis of the ATP is not known (5).

Complex I, the NADH-coenzyme Q reductase, has been shown to contain iron-sulfur proteins, flavoproteins and possibly a structural protein (6,7). Hatefi and Stempel (8) propose that in Complex I and presumably in the intact mitochondria, the NADH dehydrogenase is protected by a hydrophobic sheath of lipids and structural proteins. This review will deal primarily with the location and function of the iron-sulfur proteins (Fe-S proteins) found in Complex I.

It has recently become apparent that Fe-S proteins, also referred to as non-heme iron proteins, occur widely in plants, animals and bacteria and play a role in respiration although their mechanism of action and structure remains unknown (9). At least six different Fe-S proteins have been found in the respiratory chain (10). Even though Fe-S proteins were found in mitochondria as early as 1953, it was not until about 10 years later that the characteristic g = 1.94 electron paramagnetic resonance (EPR) signal of Fe-S protein was found in submitochondrial particles. Attempts have been made to localize the position of the Fe-S proteins in the respiratory chain and in phosphorylation reactions through their relationship to other components, EPR

spectroscopy and the site of action of specific inhibitors (9).

Study of the mechanisms involved in the respiratory chain in mammalian systems has proved very difficult. Strong and unknown forces hold the catalysts of oxidative phosphorylation tightly bound to the inner membrane of the mitochondria and separation of these components from the membrane results in alteration of their properties (11). Difficulty in separating membrane components in mammals led many investigators to turn to various yeasts to study mitochondrial and submitochondrial particles. By using a single species and specific variables any variations that might be due to differences in species can be overcome (12). The use of yeasts also allows the use of nutrient restrictions or modifications that would be unfeasible in studying the mitochondria of mammalian species. Therefore, much of the work reported in this review has been done with yeasts. Two different yeasts have been used primarily, *Candida* or *Torulopsis utilis (C. utilis)* and *Saccharomyces cerevisiae (S. cerevisiae)*. The NADH dehydrogenase from *C. utilis* has been found to resemble the mammalian systems more closely than that from other yeasts with regard to substrate specificity, electron acceptors and reactivity toward inhibitors (13).

Beinert and Sands (14) were one of the first groups to assign the characteristic EPR signal, g = 1.94, of an unidentified component of mitochondria to an iron-containing electron carrier which functioned near the flavin region of the respiratory chain. The EPR signal accounted for only a very small portion of the Fe-S proteins present in mitochondria (15). Initially, the signal was found in materials

that contained flavin which gave rise to the idea that a flavin-iron complex was responsible for the signal at g = 1.94 (15,16). The improbability of the Fe-S protein being attached to a flavin was indicated by the fact that similar signals have been found associated with Fe-S proteins that have been isolated and do not contain flavin (17). Further evidence that the Fe-S protein of NADH dehydrogenase which shows the characteristic g = 1.94 EPR signal is associated with the respiratory chain is that it appears and disappears fast enough to be consistent with participation in catalysis (18). The signal of the Fe-S proteins of the NADH dehydrogenase is distinguishable from the adjacent signals associated with the iron moieties of succinate dehydrogenase and of the cytochrome b-c region (19).

Bois and Estabrook (20) using bovine heart mitochondria suggested that the g = 1.94 signal was associated with the rotenone sensitive site. Garland (21) also showed a correlation between coupling at Site I and the presence of rotenone or piericidin A sensitivity. It has been suggested that barbiturates, amytal, rotenone, and the antibiotic, piericidin A, inhibit by blocking the reduction of NADH dehydrogenase by NADH (22). By comparing submitochondrial particles prepared from *S. cerevisiae* and *C. utilis* Sharp et al. (23) found that those from *S. cerevisiae* show no NADH induced EPR signal and no rotenone sensitivity whereas those derived from *C. utilis* do. *S. cerevisiae* lack energy conservation at Site I while *C. utilis* have the normal Sites I, II and III and the characteristic g = 1.94 signal (13). This comparison of these submitochondrial particles gave further support to the fact that the g = 1.94 signal was associated with the NADH dehydrogenase.

Many investigators (12,24-27), by preparing mitochondria from *C. utilis* cells grown in an iron-limited medium, have obtained support for the concept that Fe-S proteins, the EPR signal, and the piericidin A or a rotenone sensitive-site were related to the mechanism of energy conservation at Site I. Light et al. (12) found that *C. utilis* grown on a medium limited in iron lacked energy conservation at Site I, lacked the EPR signal, and lacked sensitivity toward piericidin A or rotenone, but still possessed the capability for electron transport. These observations with different yeasts and nutrient media led to the conclusion that the Fe-S protein involved in energy conservation at Site I was the same as the one involved in both rotenone and piericidin A sensitivity. Later investigators (24-27) using similar techniques also found absence of energy conservation at Site I and of piericidin A sensitivity. It was found that by adding small increments of iron to the medium energy conservation at Site I would occur in the absence of sensitivity towards piericidin A. A further increase in the iron in the medium was required before piercidin A or rotenone sensitivity returned. It seemed probable that the functional alterations caused by iron-limited growth were due to structural modifications or perhaps even deletions of Fe-S proteins (26).

Repetition of the iron-recovery experiment with *C. utilis* using cycloheximide, an inhibitor of cytoplasmic rather than mitochondrial protein synthesis, resulted in cells which lacked sensitivity toward piericidin A but did possess energy conservation at Site I (27). Clegg and Garland (26) speculated that the recovery of piericidin A sensitivity involved cytoplasmic protein synthesis whereas recovery

of energy conservation at Site I involved reconstitution of a Fe-S protein rather than protein synthesis.

The above experiments seemed to establish that there were at least two involvements of Fe-S proteins in the segment of the respiratory chain from NADH to the cytochromes: one involved in energy conservation and one involved in piericidin A or rotenone sensitivity (28). Since the majority of Fe-S proteins contain equal proportions of iron and labile sulfur (9), it seemed that the effects of sulfate-limited growth should be similar to those of iron-limited growth (21). Haddock and Garland (29) showed that restricting the concentration of sulfate in the medium did result in the absence of both energy conservation at Site I and piericidin A sensitivity. Increasing the sulfate concentration in the medium caused a parallel return of the two properties. Return of energy conservation at Site I and piericidin A sensitivity to sulfate-limited cells in the presence of low but increased concentrations of sulfate with or without cycloheximide was similar to that of the corresponding iron-recovery experiments.

Ohnishi et al. (24) have stated that the Fe-S protein associated with NADH dehydrogenase must be located on the substrate side of the piericidin A or rotenone sensitive site. Their proposal is supported by the fact that the EPR signal is reduced to nearly the same extent in submitochondrial particles inhibited by amytal, rotenone, or piericidin A as in untreated ones (22,24). Palmer et al. (22) proposed that the site of inhibition by amytal, rotenone, and piericidin A was between NADH dehydrogenase and coenzyme Q. More recent

investigators (30) place the site of inhibition on the O_2 side of the Fe-S protein associated with energy conservation but suggest that the binding site is part of the NADH dehydrogenase. Chance et al. (31) have proposed that the NADH dehydrogenase portion of the respiratory chain contains two flavoproteins Fp_{D_1} and Fp_{D_2} with both the rotenone sensitive site and Site I energy conservation between them as shown in Figure 2. The first flavoprotein is distinguished physically by its high fluorescence, and chemically by its low redox potential, nearly as low as that of NADH. The second flavoprotein is characterized physically by its low fluorescence efficiency, and chemically by its high redox potential (32). Serious doubts have been cast on the proposal that a second flavoprotein is present in this region of the respiratory chain by the finding that this second flavoprotein is in the soluble fraction of the mitochondria and is not present in mitochondrial preparations from all animals (33).

Many investigators have shown that the g = 1.94 EPR signal obtained at 77°K results from the Fe-S protein associated with the NADH dehydrogenase region of the respiratory chain of *C. utilis* (23,25) and mammalian (31,34) submitochondrial particles. Ohnishi et al. (35) were the first to report the presence of two different species of <77°K EPR signals arising from Fe-S proteins in the NADH dehydrogenase region of the respiratory chain in submitochondrial particles of *C. utilis*. Ohnishi et al. (36) and Orme-Johnson et al. (37) later detected similar <30°K signals in bovine heart submitochondrial particles but did not observe any of the three signals in *S. cerevisiae* submitochondrial particles which lack energy conservation at Site I.

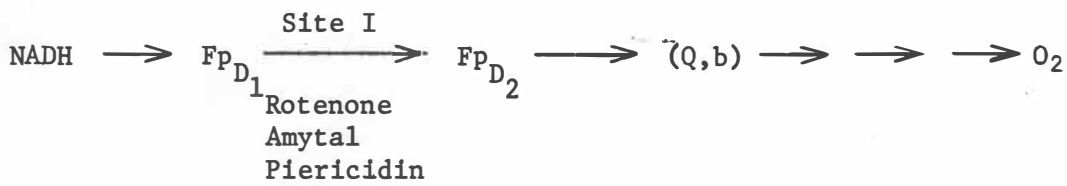

Figure 2. Tentative scheme for location of inhibitors and energy coupling at Site I.

The effect of iron concentration on the amplitude of the low temperature-detectable EPR signals in *C. utilis* was examined since it was known that the amplitude of the g = 1.94 signal is dependent on the concentration of iron in the culture medium. When the iron concentration was lowered the relative intensity of the EPR signals decreased in parallel. These observations led to the suggestion that there were at least three different species of EPR-detectable Fe-S proteins present in the NADH dehydrogenase region of the respiratory chain, possibly contained on different parts of the enzyme molecule (36).

Orme-Johnson et al. (37) later showed the presence of four Fe-S centers in Complex I from bovine heart mitochondria. These have been termed Center 1, 2, 3 and 4. Center 1 is the most labile and disappears on exposure to NADH for more than five minutes. Centers 3 and 4 cannot be differentiated from one another at the protein concentrations required for optical spectrophotometric observation. Ohnishi et al. (38) reported that similar signals in *C. utilis* were all located on the substrate side of the piericidin A inhibition site.

As the electrons are passed from one compound to another in the respiratory chain oxidation-reduction reactions are taking place with each electron donor having a characteristic electron pressure and each electron acceptor having a characteristic affinity. These electron pressures and affinities can be measured in terms of an electromotive force or potential and each electron donor when tested under standard conditions has a characteristic oxidation-reduction potential (redox potential). The thermodynamic tendency is for electrons to flow from the most negative compound, that having the highest electron pressure

to the most positive compound. The redox potentials of the main components of the respiratory chain are shown in Figure 3 (39).

The order of redox potentials for the four Fe-S centers is 1<3+4<2 (37,38,40) as shown in Figure 4. The redox potential for Center 1 was found to be -0.240 volts (v) for *C. utilis* (37) and -0.305 v for the mammalian respiratory chains (41). The redox potential for the combined Centers 3+4 is -0.210 v while that for Center 2 is -0.05 v. It is interesting to note the gap between the redox potentials of Centers 1 and 2. In the respiration of *C. utilis* using ethanol as a substrate, Center 1 is largely (approximately 80 percent) reduced while Center 2 is about 50 percent reduced. This gives a redox potential difference of approximately 0.240 v which suggests that the energy conservation for Site I phosphorylation occurs between Center 1 and Center 2 (38). This is in agreement with the earlier work done on mammalian systems (40).

The scheme for the respiratory chain in Figure 5 was drawn by the author after consideration of all the information presented in the review of the literature and represents a compilation of the work of many authors. It will later be shown that the results of this investigation show that the S-protein mentioned in the introduction was in reality an Fe-S protein involved in energy conservation at Site I.

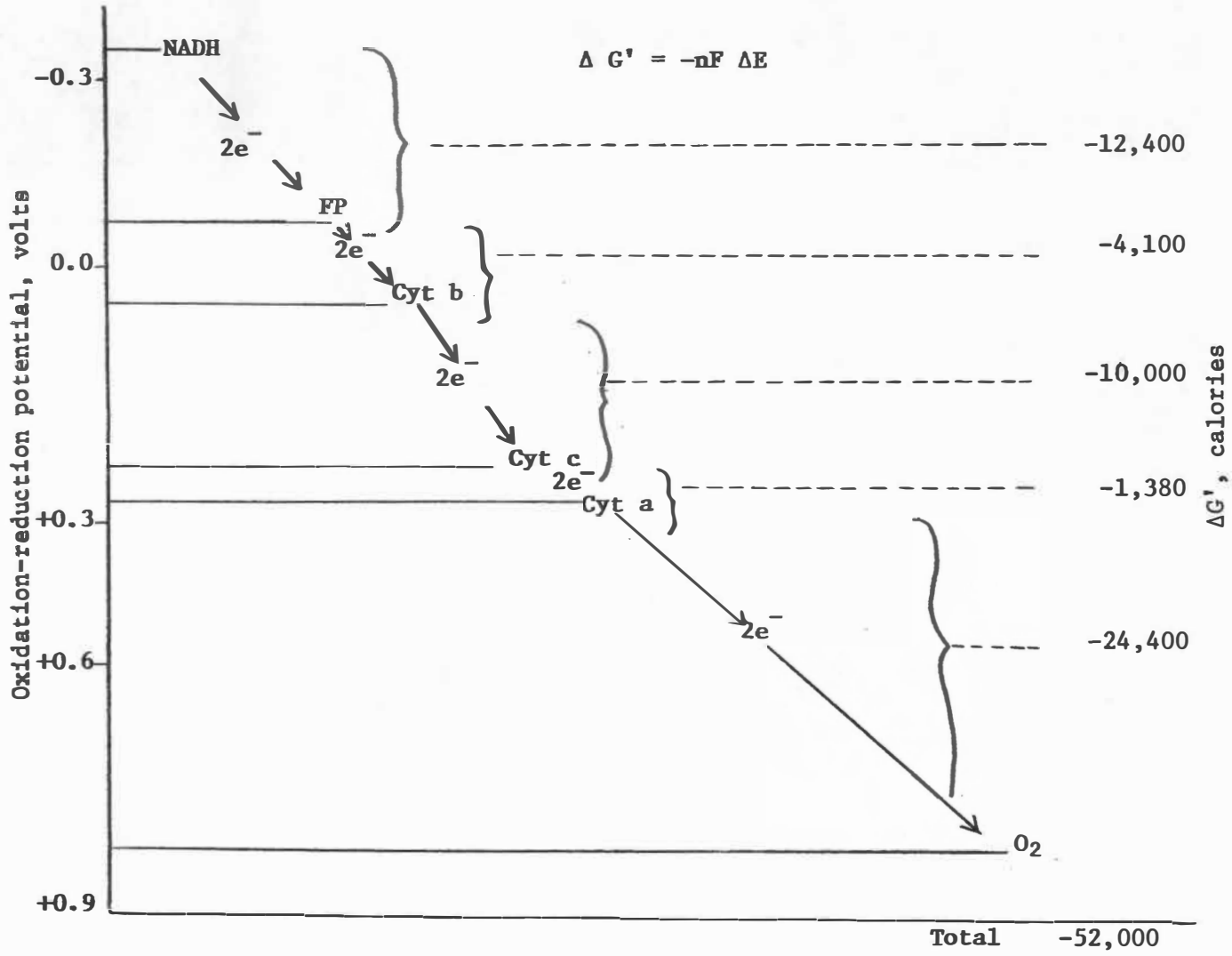

Figure 3. Relationship of electron pressure to free energy.

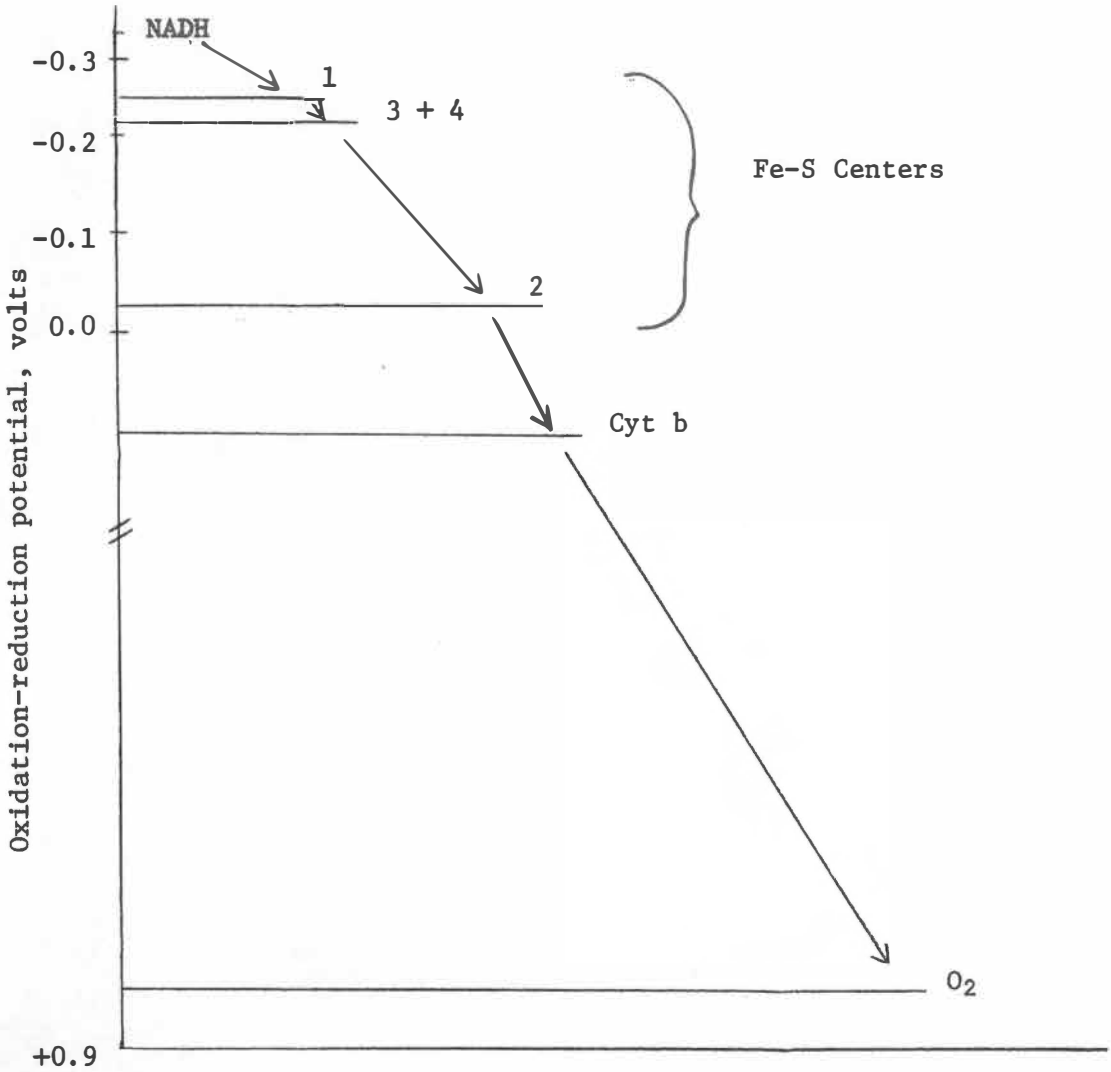

Figure 4. Schematic representation of the electron pressure of the four Fe-S Centers in the respiratory chain in *C. utilis* submitochondrial particles.

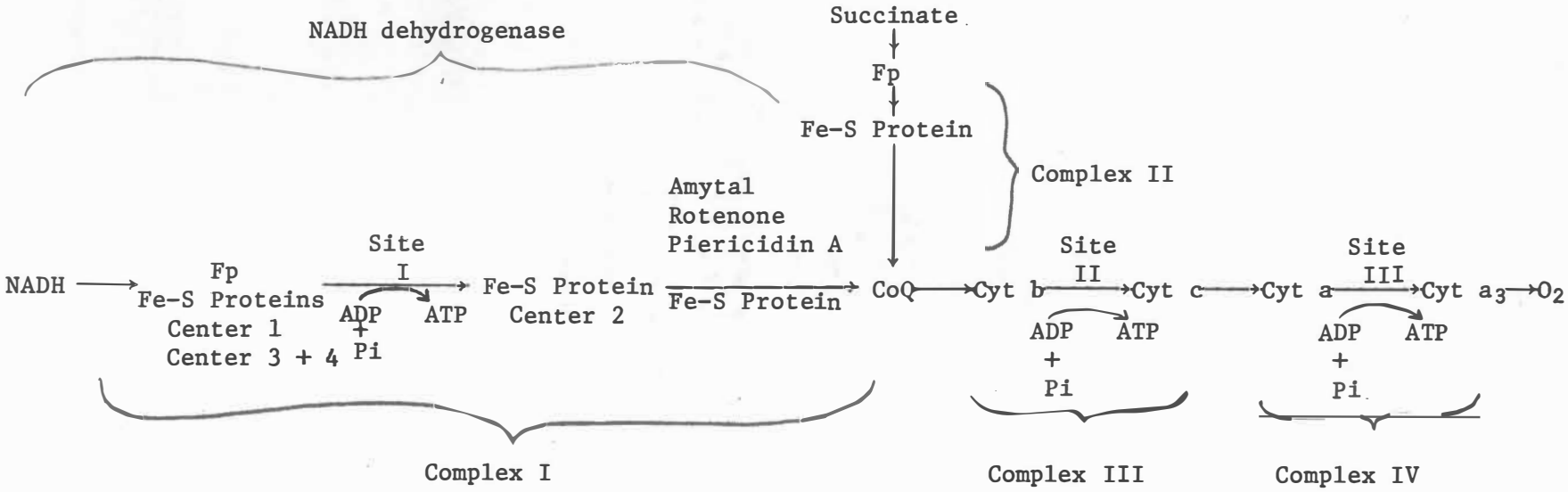

Figure 5. Proposed scheme for the mitochondrial respiratory chain.

CHAPTER III

EXPERIMENTAL PROCEDURE

A. GENERAL PLAN

Since previous investigations have shown that neither avitaminosis E (1) nor stress (2) affected the Fe-S protein, and an early experiment in this investigation demonstrated that dietary inorganic sulfate was without effect, rats fed laboratory chow[1] were used as tissue donors. The rats were sacrificed by decapitation after a stunning blow on the head and the livers removed. Livers were pooled and stored at -20° until cellular lipoprotein isolation began. The Fe-S protein was isolated from the cellular lipoprotein prepared by the salt extraction method of Smith et al. (42) as modified by Levin and Thomas (43).

Based on the rate of ^{35}S-sulfate ($^{35}SO_4^{=}$) turnover the Fe-S protein was originally thought to have a precursor relationship to other sulfur-containing compounds since it became maximally labeled sooner and lost its label earlier than any of the other fractions of the cellular lipoprotein (1). Based on this assumption earlier experiments attempted to demonstrate the transfer of $^{35}SO_4^{=}$ from the Fe-S protein to mucopolysaccharides and later to an artificial sulfate acceptor, p-nitrophenol to form p-nitrophenyl sulfate.

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[1]Ralston Purina, St. Louis, Missouri.

For ^{35}S tracer studies, the Fe-S protein was pulse labeled by a subcutaneous injection of 10 to 25 μCi carrier free $Na_2{}^{35}SO_4$[2] in 0.5 ml isotonic saline one hour prior to sacrifice since maximal labeling of the Fe-S protein occurred one hour following injection of a test dose of $^{35}SO_4^{=}$ (1). Radioactivity of the samples was determined using a Picker Nuclear Liquimat 220 Liquid Scintillation Counter.

Since transfer of $^{35}SO_4^{=}$ from the Fe-S protein was inhibited by ATP and no transfer could be demonstrated with the artificial sulfate acceptor, p-nitrophenol, it was postulated that the Fe-S protein might be a high energy compound involved in the oxidative phosphorylation of ADP. Based on this assumption experiments were conducted to 1) detect a disulfide configuration, 2) determine the iron concentration, 3) determine the effect of an uncoupling agent, 4) establish esterification of inorganic phosphate, and 5) determine the uptake of $^{32}PO_4^{\equiv}$ by ADP as influenced by the Fe-S protein.

B. METHODS

Diets

The composition of the diets is shown in Table 1. In the first experiment three groups of five male rats were fed the 0.0002 percent, the 0.10 percent and the 0.42 percent inorganic sulfate diets. In all the experiments using 2,4 dinitrophenol the 0.02 percent inorganic sulfate diet was used.

---

[2]New England Nuclear, Boston, Mass.

TABLE 1

COMPOSITION OF DIETS

	0.0002% $SO_4^{=}$	0.02% $SO_4^{=}$	0.10% $SO_4^{=}$	0.42% $SO_4^{=}$
		g/100 g		
Casein	15.00	15.00	15.00	15.00
Cornstarch	30.00	30.00	30.00	30.00
Sucrose	30.00	30.00	30.00	30.00
Cod Liver Oil	2.00	2.00	2.00	2.00
Vegetable Shortening[1]	6.00	6.00	6.00	6.00
Vitamin mixture[2]	2.00	2.00	2.00	2.00
Non-nutritive bulk[3]	11.79	11.68	11.74	12.00
Basic salt mixture[4]	1.34	1.34	1.34	1.34
DL methionine[3]	0.66	0.62	0.50	00
$CaCO_3$	1.34	1.32	1.24	0.91
$CaSO_4 \cdot 2\ H_2O$	00	0.04	0.18	0.75

[1]Crisco, Proctor and Gamble, Cincinnati, Ohio.

[2]Nutritional Biochemicals Corporation, Cleveland, Ohio. Vitamin diet fortification mixture formulated to supply the following amounts of vitamins (g/kg vitamin premix): thiamine hydrochloride 1.0, riboflavin 1.0, niacin 4.5, p-aminobenzoic acid 5.0, calcium pantothenate 3.0, pyridoxine hydrochloride 1.0, ascorbic acid 45.0, inositol 5.0, choline chloride 75.0, menadione 2.25, biotin 0.020, folic acid 0.090, vitamin B_{12} 0.00135, α-tocopherol 5.0, vitamin A 9 x 10^5 units, vitamin D 1 x 10^5 units, and sufficient glucose to make 1 kg.

[3]Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[4]1.34 g equals in mg: 92.8 $MgCO_3$, 207.0 NaCl, 336.0 KCl, 636.0 KH_2PO_4, 61.5 $FePO_4 \cdot 2H_2O$, 0.2 KI, 0.3 NaF, 1.8 $AlK(SO_4)_2 \cdot 12\ H_2O$, 2.1 $Cu(C_2H_3O_2) \cdot H_2O$, 1.2 $MnCl_2 \cdot 6\ H_2O$. Hubbell et al. (44) as modified by Pendergrass (45).

Isolation of Cellular Lipoprotein

The livers were removed from the freezer and allowed to thaw at 4° overnight. Approximately 50 g of liver were homogenized in cold Solution I using a motor-driven Thomas size C homogenizer with a teflon pestle. Solution I is a 0.16 M KCl solution each liter containing 186 mg of iodoacetic acid, as an enzyme inhibitor, and 5 g of sodium citrate, to prevent clotting of any blood present, and the final solution adjusted to pH 4.7 with concentrated HCl. The homogenate was transferred to a 100 ml ground glass stoppered mixing cylinder, diluted to 100 ml with Solution I and the contents mixed by inversion. All solutions were kept between 3° and 5° throughout the entire isolation procedure. The homogenate was centrifuged at 28,000 x g for 40 minutes in a Lourdes "Beta-fuge" using a 9RA head at 4°. The supernatant fluids were discarded and the centrifuge cake homogenized again with Solution I and centrifuged. The centrifuge cake was then homogenized with Solution III (a 0.4 M KCl solution containing 186 mg of iodoacetic acid and 10 g of sodium citrate per liter) and centrifuged. The homogenizing of the centrifuge cake with Solution III and the centrifugation were repeated. Next the centrifuge cake was homogenized with Solution II (a 1.0 M KCl solution containing 186 mg of iodoacetic acid and 10 g of sodium citrate per liter, the final solution was adjusted to pH 4.7 by the addition of concentrated HCl) and centrifuged. The procedure was repeated making the volume of the homogenate up to 80 ml each time and alternating between Solutions II and III until the supernatant fluid gave a negative protein test with 10 percent trichloroacetic acid (TCA). In order

to lower the salt concentration of the centrifuge cake, it was washed with a 1:1 aqueous dilution of Solution I and centrifuged again. The resulting centrifuge cake of insoluble cellular lipoprotein was homogenized with 5 ml of cold acetone per g of liver, the resulting mixture filtered by suction filtration, and the residue allowed to air dry and weighed.

Fractionation of Cellular Lipoprotein

Lipid was extracted from the cellular lipoprotein by heating with 20 ml 2:1 chloroform:methanol per g in a 60° water bath for two hours. The resulting residue was separated by suction filtration, washed with 5 ml 2:1 chloroform:methanol per g and allowed to dry.

To remove the mucopolysaccharides, the air-dried residue was stirred with 10 ml of 0.5 M KSCN per g for three hours. For each gram of sample, 5 ml 80 percent phenol in distilled water were added and the stirring was continued for an additional four hours. The mixture was allowed to stand overnight after which the emulsion was separated by centrifugation at 715 x g in an International Model SBV centrifuge for 10 minutes. The centrifuge cake was washed once with 5 ml of 0.5 M KSCN per g of original residue. The emulsion was again separated by centrifugation at 715 x g for 10 minutes and the supernatant fluid poured off.

The protein was removed from the phenol phase after removal of the 0.5 M KSCN supernatant fluid by the addition of five volumes of acetone. The protein which was precipitated was removed by centrifugation at 715 x g for 10 minutes and dried with acetone.

The nucleotides were then extracted by heating the residue with 10 ml of 5 percent TCA per 200 mg in a 90° water bath for 30 minutes. The TCA-extracted residue was collected by suction filtration and dried with acetone. Sufficient 2 N NaOH was added to adjust the pH to 8 as was determined by using pHydrion paper (A).[3] Enough 0.5 N NaOH was added to yield a final concentration of 25 ml per gram of residue. The mixture was shaken at 0 to 5° for 16 hours. The pH was adjusted to pH 7.0 with glacial acetic acid and the residue removed by centrifugation at 715 x g for 10 minutes. The residue was washed and again removed by centrifugation at 715 x g for 10 minutes. The supernatant fluids were combined with four volumes of ethanol and allowed to precipitate overnight at -20°. The precipitate (Fe-S protein) was collected by centrifugation at 715 x g for 10 minutes and washed three times with ethanol. For all the $^{35}SO_4^{=}$ transfer studies the precipitate was dried with ether and the specific activity determined.

For studies in which the Fe-S protein was being studied as a high energy compound, the precipitate was further purified. The ethanol precipitate was weighed and dissolved in 5.0 ml of 0.1 M imidazole buffer, pH 9.2, per g wet weight. The resulting mixture was centrifuged at 715 x g for 10 minutes and the supernatant fluid dialyzed against running water overnight. The resulting dialysate was centrifuged at 715 x g for 10 minutes and the supernatant fluid shell-frozen in an acetone-dry ice mixture and lyophilized. The dried

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[3]Scientific Products, Evanston, Illinois.

product was weighed and stored at 4° until used. This product will be hereafter referred to as "purified" Fe-S protein.

Determination of $^{35}SO_4^{=}$ in the Fe-S Protein and in Mucopolysaccharides

The specific activity of the $^{35}SO_4^{=}$ was determined according to the method of Mahin and Lofberg (46). Approximately 2 to 3 mg samples of either the Fe-S protein or the mucopolysaccharides were transferred to counting vials. All samples were analyzed in duplicate. To each vial were added 0.2 ml distilled water, 0.2 ml of 70 percent $HClO_3$ and 0.4 ml H_2O_2. The samples were heated in a 70-80° water bath for approximately 30 minutes or until the material had dissolved. The samples were allowed to cool to room temperature and a scintillation cocktail composed of 6.0 ml of 2-ethoxyethanol and 10 ml of PPO[4] (2,5 diphenyloxazol) in toluene made by adding 12 g PPO per liter of toluene. There was no appreciable quenching as indicated by the channels ratio data.

Transfer of $^{35}SO_4^{=}$ From the Fe-S Protein to Mucopolysaccharides

The Fe-S protein as prepared for the $^{35}SO_4^{=}$ transfer experiments was used. If a radioactive fraction was not required the Fe-S protein was isolated by the same procedure from rats that had not received a $^{35}SO_4^{=}$ injection. Mucopolysaccharides were isolated by the method of Bostrom (47) from the rib cartilage of those rats that had not received $^{35}SO_4^{=}$. As a source of enzymes, a 10 percent liver homogenate was prepared in 0.067 M phosphate buffer, pH 7.4 and centrifuged at 650 x g for 10 minutes to remove the nuclei.

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[4]Packard Instrument Company, Inc., Downers Grove, Illinois.

Mixtures containing 1.0 ml of the enzyme solution either fresh or boiled were incubated at 37° for one hour in Warburg flasks without center wells with air as the gas phase. The complete incubation mixture contained 40 mg of Fe-S protein [4000 counts per minute (cpm)] added as 0.5 ml of a solution prepared by dispersing 200 mg of Fe-S protein in 2 ml of 0.067 M K_2HPO_4 and adding 0.5 ml of 0.067 M KH_2PO_4 to achieve a solution with a pH of 7.4. Mucopolysaccharides were added as 0.5 ml of a solution containing 30 mg of mucopolysaccharides dissolved in 1 ml of 0.067 M phosphate buffer, pH 7.4. $MgCl_2$ and Na_2SO_4 were added as 0.1 ml each of a 0.15 M solution. Total volume of solution in the flask was adjusted to 3 ml by addition of 0.067 M phosphate buffer, pH 7.4. The variations in the incubation mixture were: 1) no added ATP, 2) no added mucopolysaccharides, 3) no added Fe-S protein, and 4) boiled enzyme. At the end of the incubation period the reaction was stopped by the addition of 2 ml of 10 percent TCA. The precipitated Fe-S protein was removed by centrifugation at 715 x g for 10 minutes and the mucopolysaccharides precipitated from the supernatant fluid by the addition of four volumes of ethanol. The precipitate was collected by centrifugation at 715 x g for 10 minutes and purified by dissolving in 0.5 N NaOH and reprecipitated with four volumes of ethanol. The specific activity of the isolated mucopolysaccharides was determined.

Transfer of $^{35}SO_4^{=}$ from the Fe-S Protein to p-Nitrophenol Using Sulfotransferase

Sulfotransferase was prepared according to the method of Hilz

and Lipmann (48). A 25 percent homogenate of fresh rat liver was made using a 0.25 M sucrose solution containing 0.08 M $KHCO_3$. The homogenate was centrifuged at 510 x g for 15 minutes in the cold (4°). One-tenth of its volume of 1.5 M KCl was added with stirring to the supernatant fluid containing the mitochondria. The mitochondria were removed by centrifuging at 14,750 x g for one hour in the cold. To obtain a clear and more active preparation the cloudy supernatant fluid was recentrifuged for one hour at 28,000 x g in the cold.

Six hundred fifty ml of clear liver supernatant fluid plus 100 ml of Alumina C_γ gel were stirred for 30 minutes and then centrifuged at 1200 x g for 20 minutes in the cold. The gel was extracted with 1 liter of a 0.20 percent saturated $(NH_4)_2SO_4$ solution, pH 7.2. To the extract a solution of 4 M [saturated] $(NH_4)_2SO_4$, pH 7.0, containing 0.002 M versene was added to a final saturation of 0.520 and the precipitate collected and dissolved in 0.02 M Tris-HCl buffer, pH 7.0.

One hundred fifty milliliters of the solution were diluted with an equal volume of cold water and refractionated between 0.20 and 0.35 percent saturation of $(NH_4)_2SO_4$. The precipitate was dissolved in 25 ml 0.02 M Tris-HCl buffer, pH 7.0. The enzyme solution was dialyzed for 2 hours at 4° against 0.01 M Tris-HCl buffer, pH 7.4, to free it of sulfate.

A modification of the method of Levi et al. (49) was used in the transfer of $^{35}SO_4^{=}$ to p-nitrophenol. Mixtures containing either 0.5 or 1.0 ml of the enzyme solution were incubated at 37° for 40 minutes in Warburg flasks without center wells or side arms with air

as the gas phase. The complete incubation mixture contained 10 mg of Fe-S protein added as 0.5 ml of a solution prepared by dispersing 70 mg of Fe-S protein in 4 ml of 0.02 M Tris-HCl buffer, pH 7.0. All flasks contained 0.00112 M p-nitrophenol added as 0.04 ml of solution. The variations included the use of two different enzyme levels, 0.5 ml and 1.0 ml with a boiled enzyme preparation used as a control. The other variation included the addition of 0.28 M ATP[5] and 0.14 M $MgCl_2$ both as 0.04 ml of solution. The total volume of solution in the flask was adjusted to 1.54 ml by the addition of 0.02 M Tris-HCl buffer, pH 7.0. At the end of the incubation period, the reaction was stopped by the addition of 3 ml of 95 percent ethanol, and the mixture freed of the precipitated protein by centrifugation at 715 x g for 10 minutes. The total supernatant fluid for each flask was stripped on a separate chromatogram using Whatmann #1 filter paper. The chromatograms were developed using 9:1 ($^{v}/v$) acetone:water as the solvent. p-Nitrophenyl sulfate was eluted from the solvent front with 9:1 ($^{v}/v$) acetone:water by descending chromatography. The eluates were placed in liquid scintillation vials, concentrated and 16 ml of a scintillation cocktail, composed of 6 ml of 2-ethoxyethanol and 10 ml of PPO in toluene added and the mixture counted. The strip along the origin containing the residual sulfate was removed, the sulfate eluted with water, concentrated, and counted.

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[5]Sigma Chemical Company, St. Louis, Missouri.

Determination of the Presence of a Disulfide Configuration in the Fe-S Protein

Each tube contained 3 mg of "purified" Fe-S protein added as 2 ml of a solution prepared by dispersing 24 mg in 14 ml of 0.28 M Tris-HCl buffer, pH 7.4. Duplicate tubes contained 1 mmole of cysteine added in 1 ml of 0.28 M Tris-HCl buffer, pH 7.4 and two tubes contained 1 mmole of 2-mercaptoethanol added in 1 ml of 0.28 M Tris-HCl buffer, pH 7.4. The control tubes contained only 1 ml of 0.28 M Tris-HCl buffer, pH 7.4 and 2 ml of Fe-S protein suspensions. The mixtures were allowed to stand at room temperature for 1 hour and the contents of the tubes transferred to 1/4 inch dialysis bags and dialyzed against distilled water for nine hours. The distilled water was changed twice and the dialyzing mediums were combined in a 50 ml centrifuge tube. One ml carrier sulfate (2.85 ml H_2SO_4/liter) was added and the sulfate precipitated with 1 ml of 1 percent benzidine-HCl in ethanol. The solution was centrifuged at 715 x g for 10 minutes and the precipitate combusted with 0.2 ml of 70 percent $HClO_3$ and 0.4 ml of 30 percent H_2O_2. The contents of the centrifuge tube were transferred to a scintillation vial and counted.

Determination of the Iron Content of the Fe-S Protein

Approximately 100 mg of the "purified" Fe-S protein were placed in a 30 ml micro-Kjeldahl flask with 1 ml of a 7 + 1 v/v $HClO_3$-H_2SO_4 mixture and 4 ml of concentrated HNO_3. The mixture was digested on electric heaters, adding extra HNO_3 when necessary, until only the H_2SO_4 and inorganic constituents of the material remained. The

contents of the flask were cooled, 1.25 ml of demineralized water added and the contents filtered into 10 ml volumetric flasks. The contents of the micro-Kjeldahl flask were quantitatively transferred to volumetric flasks and the flasks made to volume with distilled water. Two reagent blanks were also prepared.

Working standard iron solutions were prepared from a concentrated stock iron solution. The optimum range suggested is 2 to 20 parts per million (ppm) (50). Percent absorption of the samples and standards was determined using a Perkin-Elmer Atomic Absorption Spectrophotometer 303. The energy source was a hollow cathode tube and an acetylene and air flame was used with a Boling burner head.

Determination of the Effect of an Uncoupling Agent, 2,4 Dinitrophenol, on the Incorporation of ^{35}S-sulfate into the Fe-S Protein

2,4 Dinitrophenol, an uncoupling agent, was added (50 mg/100 g diet) to a 0.02 percent sulfate diet (Table 1, page 19) and fed to 11 adult male rats. The control rats received the 0.02 percent sulfate diet without added 2,4 dinitrophenol. The rats were housed in group cages and given feed and water ad libitum. On the fifth day all rats were pulse labeled with a subcutaneous injection of 10 - 25μCi of carrier free $Na_2{}^{35}SO_4$ in 0.5 ml isotonic saline and all animals sacrificed one hour after injection. The livers were removed, transferred to cold Solution I and the Fe-S protein prepared as described previously. The resulting product was weighed and counted.

Determination of $^{59}Fe^{++}$ and $^{35}SO_4^{=}$ in the Fe-S Protein Isolated from 2,4 Dinitrophenol-Treated Rats

As in the previous experiment the 0.02 percent sulfate diet

containing 2,4 dinitrophenol (50 mg/100 g diet) was used and fed to eight rats. On the fourth day all rats were injected with 25 μCi carrier free $Na_2{}^{35}SO_4$ and 25 μCi $^{59}FeSO_4$[6] in 0.5 ml isotonic saline and sacrificed one hour after injection. The livers were removed and transferred to cold Solution I and the Fe-S protein prepared as described previously. The resulting product was weighed and counted. The counts attributable to sulfate were determined as follows.

An approximate 1.0 mg sample of the Fe-S protein was combined with approximately 800 mg benzoic acid to form a pellet which was combusted in a Parr bomb. After combustion the samples were transferred to a 50 ml centrifuge tube and 1 ml of carrier sulfate added. The sulfate was precipitated with 1 ml of 1 percent benzidine-HCl in ethanol. The precipitate was collected by centrifugation at 715 x g for 10 minutes, combusted and counted. The counts attributable to iron were determined by difference.

Determination of the Esterification of Inorganic Phosphate

"Purified" Fe-S protein added as 20 mg of Fe-S protein per 0.25 ml of 0.1 M imidazole buffer, pH 7.4, was incubated with 20 μmoles of ADP[7] and 20 μmoles of phosphate added as 0.5 ml of 0.1 M imidazole buffer, pH 7.4. Two different levels of the Fe-S protein were used, 20 mg and 40 mg. Other variations included no added ADP, no added Fe-S protein or the addition of 2 ml of 10 percent TCA.

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[6] Schwarz/Mann, Orangeburg, New York.

[7] Sigma Chemical Company, St. Louis, Missouri.

The mixtures were incubated at 37° for 40 minutes in Warburg flasks without side arms or center wells with air as the gas phase. The reaction was stopped by the addition of 2 of 10 percent TCA. The contents of the Warburg flasks were filtered into 25 ml volumetric flasks and the contents adjusted to volume with distilled water. Duplicate 1 ml aliquots were placed in 10 volumetric flasks for phosphate determination.

Phosphorus was determined according to the method of Allen (51). Duplicate standards were prepared by placing 2.5 ml of a stock solution containing 0.01 mg P per in a 10 ml volumetric flask. A reagent blank was also prepared. To each of the flasks were added 1 of 70 percent $HClO_3$, 1 ml hydroquinone reagent (0.25 g hydroquinone and 5 g of pure $NaHSO_3$ per 25 ml water) and 0.5 ml of 8.3 percent ammonium molybdate in that order. The samples were made to volume with distilled water and after 5-30 minutes the absorbance determined at 650 nanometers (nm) with a Beckman B spectrophotometer.

Determination of the Incorporation of ^{32}P into $AT^{32}P$

Pressman's method (52) was used to determine the incorporation of ^{32}P into $AT^{32}P$. To 0.5 ml of 0.1 M imidazole buffer, pH 7.4, containing 40 ml of "purified" Fe-S protein, 20 μmoles of ADP and 20 μmoles phosphorus, was added a neutralized solution (neutralized with KOH) of ^{32}P[8] containing 5.5 x 10^5 cpm. Additional samples contained 200 μmoles $MgCl_2$. The solutions were incubated at 37° in Warburg flasks without center wells or side arms with air as the gas phase. The reaction

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[8]Obtained as $H_3{}^{32}PO_4$ from Amersham Searle Corp., Arlington Heights, Illinois.

was allowed to proceed with gentle shaking for either 20, 45 or 90 minutes. The reaction was stopped with 2 ml of 10 percent TCA and the precipitate removed by centrifugation at 715 x g for 10 minutes. The supernatant fluid was decanted and the precipitate washed with three separate washings of 1 ml of distilled water. The supernatant fluids were combined, 5 μmoles of carrier nucleotide added to the reaction mixture and the volume made up to 10 ml before placing it on the column. The column was prepared using purified Dowex-1-Cl, X-8, 200-400 mesh from the Bio-Rad Company and converted to the formate form by washing with 3 M sodium formate. Resin columns 1 cm in diameter and 3 cm high were used. The supernatant fluid was allowed to pass into the resin column and the column washed with two 15 ml portions of distilled water. The ^{32}P and ADP were removed with three 15 ml washes of 4 M formic acid in 0.05 M ammonium formate and the $AT^{32}P$ eluted with two 15 ml portions of 4 M formic acid in 0.5 M ammonium formate. Two ml of the eluate containing the $AT^{32}P$ were counted using a scintillation cocktail containing 5.0 g permablend and 120 g of napthalene per liter of dioxane.

Determination of the Purity of $AT^{32}P$

Purity of the $AT^{32}P$ was determined according to the method of Lowenstein (53). A sample collected after washing the column with 4 M formic acid in 0.5 M ammonium formate was applied to Whatmann #3 filter paper along with a marker spot of ATP. The chromatograph was developed using ascending chromatography with the solvent composed of 46.8 ml isopropanol, 2.6 ml formic acid, 24.6 ml of 95 percent ethanol, 26.1 ml water and 20 ml pyridine. The paper was dried in a current of air

at room temperature. The portion of the chromatogram containing the $AT^{32}P$ was identified by the quenching of the ultraviolet light by the marker spot. This portion of the chromatogram was cut out, placed in a scintillation vial with 20 ml of the dioxane cocktail and counted.

CHAPTER IV

RESULTS

Previous experiments (1,2) have demonstrated that neither avitaminosis E or the stress of malathion intoxication affects the uptake of a test dose of $^{35}SO_4^{=}$ by the S-protein. However, because of the apparent precursor relationship demonstrated by the timed experiments of Fulton and Smith (1) and previous investigation in this laboratory (54-56) with respect to the effect of dietary inorganic sulfate, it seemed necessary to determine the effect of dietary inorganic sulfate on the uptake of a test dose of $^{35}SO_4^{=}$. The data which are shown in Table 2 show that, as was true for avitaminosis E and stress, variations in dietary inorganic sulfate were without effect upon the uptake of $^{35}SO_4^{=}$ by S-protein. Since these data and earlier experiments (1,2) demonstrated that S-protein was a high priority protein with respect to sulfate and was not affected by diet, rats fed laboratory chow were used as tissue donors in all future experiments.

Although an intestinal mucosa sulfate reductase has been identified (57) and the incorporation of sulfate into taurine demonstrated (58), it is generally assumed that if ^{35}S-sulfur is administered as $^{35}SO_4^{=}$, it will be incorporated as ester sulfate. Therefore, the most obvious acceptor for the sulfate of S-protein to test the apparent precursor relationship demonstrated earlier (1) is mucopolysaccharide. The data which are presented in Table 3 show the results of two experiments in which the S-protein, pulse labeled in vivo by a subcutaneous

TABLE 2

EFFECT OF DIETARY INORGANIC SULFATE ON THE UPTAKE OF $^{35}SO_4^{=}$ BY THE S-PROTEIN

Percent Sulfate in Diet	$^{35}SO_4^{=}$ Activity in S-Protein
	cpm/mg
0.0002	91[1]
0.10	90
0.42	97

[1]Values are averages of duplicate determinations from five pooled livers.

TABLE 3

IN VITRO TRANSFER OF $^{35}SO_4^{=}$ FROM IN VIVO LABELED ^{35}S S-PROTEIN TO MUCOPOLYSACCHARIDES

System	Total cpm Exp. 1	Exp. 2
Complete[1]	426[2]	396[2]
Without added ATP	547	545
Without added mucopolysaccharides	69	64
Boiled enzyme	414	333

[1]1 ml of enzyme solution, 7.5 μmoles ATP, 15 μmoles $MgCl_2$, 15 mg mucopolysaccharides, 40 mg ^{35}S S-protein (400 cpm), 2.9 ml 0.67 M PO_4 buffer, pH 7.4.

[2]Values are averages of duplicate determinations.

injection of $^{35}SO_4^{=}$ was used to transfer $^{35}SO_4^{=}$ to mucopolysaccharides in vitro. These data indicate that S-protein will transfer $^{35}SO_4^{=}$ to mucopolysaccharides when S-protein is labeled by injecting the animal with $^{35}SO_4^{=}$. Although transfer was obtained with a boiled enzyme, a slight enzyme effect was noted.

These data have demonstrated that the S-protein could transfer sulfate if labeled in vivo; however, it seemed reasonable to determine if S-protein could raise $^{35}SO_4^{=}$ to the energy level of ester sulfate if it were incubated in a medium containing $^{35}SO_4^{=}$ and an enzyme preparation from liver. These data which are shown in Table 4 show an enzyme dependent "activation" of sulfate. In addition these data as well as the data shown in Table 3 show that the addition of ATP is apparently inhibitory either to the transfer (Table 3) or the activation and transfer of sulfate (Table 4).

Although the results from previous experiments appeared to be clear cut and indicate that S-protein could raise sulfate to the energy level of ester sulfate and transfer it to mucopolysaccharides, questions arose because the acceptor could not be isolated in a highly purified state and the radioactivity associated with the mucopolysaccharides might only represent coprecipitation. It is difficult to understand how coprecipitation might explain the data obtained without added S-protein (Table 4). However, in order to answer these questions it was decided to repeat the sulfate transfer experiment using p-nitrophenol, an artificial sulfate acceptor. These data which are shown in Table 5 show little transfer of $^{35}SO_4^{=}$ from S-protein to p-nitrophenol and no enzyme effect. Those samples which contained

TABLE 4

INFLUENCES OF S-PROTEIN ON THE IN VITRO UPTAKE OF $^{35}SO_4^{=}$ BY MUCOPOLYSACCHARIDES

System	μmoles $^{35}SO_4^{=}$ in mucopolysaccharides Exp. 1	Exp. 2
Complete[1]	3.5[2]	5.1[2]
Without added ATP	6.1	5.4
Without added mucopolysaccharides	0.2	0.2
Without added S-protein	1.0	0.4
Boiled enzyme	2.8	1.4

[1] 1 ml of enzyme solution, 7.5 μmoles ATP, 15 μmoles $MgCl_2$, 15 mg mucopolysaccharides, 40 mg S-protein, 15 μmoles $^{35}SO_4^{=}$ (8000 cpm), 2.8 ml 0.067 M PO_4 buffer, pH 7.4.

[2] Values are averages of duplicate determinations.

TABLE 5

IN VITRO TRANSFER OF $^{35}SO_4^{=}$ FROM S-PROTEIN TO p-NITROPHENOL

System	Residual $^{35}SO_4^{=}$	p-Nitrophenyl Sulfate
	cpm x 10^{-2}	Total cpm
Complete[1]	15.9[2]	26
With 1 ml sulfotransferase	11.5	24
Boiled enzyme	17.4	25
With ATP and $MgCl_2$ added[3]	20.0	25

[1]0.5 ml sulfotransferase solution, 10 mg ^{35}S S-protein (9000 cpm), 0.04 μmoles p-nitrophenol, 0.5 ml 0.02 M Tris-HCl buffer, pH 7.0

[2]Values are averages of duplicate determinations.

[3]11.2 μmoles ATP and 5.6 μmoles $MgCl_2$.

the sulfotransferase enzyme preparation gave less ^{35}S activity in the p-nitrophenyl sulfate fraction than any of the others. None of the fractions incorporated more than 0.3 percent of the available activated sulfate. Therefore, it was concluded that S-protein was not effective in transferring sulfate to the artificial sulfate acceptor, p-nitrophenol.

These data opened to question the role of S-protein as an intermediate in the sulfation of those cellular constituents that contained ester sulfates. Therefore, some other function for S-protein was sought. The next step then appeared to be a check on the nature of the linkage of the sulfate to the S-protein. One way which the sulfate could attach to the S-protein would be by splitting out hydrogen between a sulfhydryl and a sulfate to form -thiolpyro-sulfate. Since this linkage was formed by oxidation, it could be cleaved by reducing agents especially sulfhydryl containing reducing agents in a modified sulfhydryl-disulfide exchange reaction. The data which are shown in Table 6 show the effect of two sulfhydryl reducing agents cysteine and 2-mercaptoethanol on the release of $^{35}SO_4^{=}$. Cysteine was not as effective in removing sulfate, however, cysteine is not as good a reducing reagent as 2-mercaptoethanol and may have been partially oxidized as purchased.

Gutman et al. (59) have demonstrated the participation of SH groups in the energy conservation reactions of a cell and suggest that the equation $2SH \rightarrow -S-S + H_2$ may function as a catalyst in energy conservation reactions. Therefore, since the S-protein had been demonstrated to undergo an apparent modified sulfhydryl-disulfide

TABLE 6

INFLUENCE OF REDUCING AGENTS ON REMOVAL OF $^{35}SO_4^{=}$ FROM THE S-PROTEIN

Reducing Agent	Total cpm in Dialyzing Medium
None[1]	44[2]
1 mmole cysteine	48
1 mmole 2-mercaptoethanol	110

[1]All systems contained 3 mg ^{35}S S-protein (3800 cpm) and 3 ml of 0.28 M Tris-HCl buffer, pH 7.4.

[2]Values are averages of duplicate determinations.

exchange reaction, it was assumed that the S-protein might have a role in energy conservation by the cell. This supposition was strengthened by the determination of iron in the S-protein. Atomic absorption spectrophotometric determinations of Fe in the S-protein demonstrated that there were approximately 0.6 μmoles of Fe/mg S-protein in the S-protein samples. The S-protein will hereafter be referred to as Fe-S protein.

As has been stated previously (37,38) three Fe-S proteins are believed to be involved with the NADH dehydrogenase complex and at least two of these participate in energy conservation at Site I (38). If the Fe-S protein is participating in energy conservation, it is conceivable that if the Fe-S protein were isolated from two groups of rats given a test dose of $^{35}SO_4^{=}$ with one group being fed 2,4 dinitrophenol, there should be a difference in the uptake of $^{35}SO_4^{=}$ between the two groups. The data which are presented in Table 7 show a 40 percent increase in the specific activity of the Fe-S protein isolated from rats fed 2,4 dinitrophenol. Although not unequivocal, these data indicate that the Fe-S protein is indeed involved in energy conservation reactions of the cell.

These data raise an important question, that is, does the apparent 40 percent increase in specific activity of the Fe-S protein as a result of 2,4 dinitrophenol treatment represent increased de novo synthesis of Fe-S protein or the filling of more than one active site on an existing Fe-S protein with the $^{35}SO_4^{=}$. Both $^{59}Fe^{++}$ and $^{35}SO_4^{=}$ were injected into a group of eight adult male rats and their Fe-S protein isolated and the specific activity of both $^{59}Fe^{++}$ and $^{35}SO_4^{=}$ determined. These data (Table 8) show an approximately equal uptake of $^{59}Fe^{++}$ and

TABLE 7

INFLUENCE OF 2,4 DINITROPHENOL ON INCORPORATION OF $^{35}SO_4^{=}$ INTO THE Fe-S PROTEIN

Dietary Additions	^{35}S Activity Fe-S Protein
Per 100 g	cpm/mg[1]
None	305 ± 27
50 mg 2,4 Dinitrophenol	429 ± 38

[1]Values are averages of three groups of six pooled livers ± standard error of the mean.

TABLE 8

THE UPTAKE OF A TEST DOSE OF $^{35}SO_4^{=}$ AND $^{59}Fe^{++}$ BY Fe-S PROTEIN

Radioactivity in Fe-S Protein	$^{35}SO_4^{=}$	$^{59}Fe^{++}$
cpm/mg[1]	203	210
Total cpm as percent of dose	8.6×10^{-1}	8.9×10^{-1}

[1]Values are averages of duplicate determinations from eight pooled livers.

$^{35}SO_4^{=}$. As stated previously (25) iron uptake by the Fe-S proteins involved in energy conservation at Site I represents de novo synthesis. Therefore, these data (Table 8) and those data obtained from animals fed 2,4 dinitrophenol (Table 7) indicate that the rat is synthesizing additional quantities of the Fe-S protein involved in energy conservation at Site I to compensate for the effect of the uncoupling of oxidative phosphorylation.

These data allow the assumption that the Fe-S protein is the high energy configuration generated just prior to the esterification of phosphate. This assumption was checked by incubating Fe-S protein with inorganic phosphate and ADP. The data obtained by incubating Fe-S protein with inorganic phosphate and ADP and measuring the disappearance of inorganic phosphate are shown in Table 9. These data show that Fe-S protein can catalyze the esterification of inorganic phosphate with ADP as an acceptor and that the esterification parallels the amount of Fe-S protein in the incubation mixture. Although these data indicate that ADP is accepting inorganic phosphate, it is conceivable that the inorganic phosphate might be bound to the Fe-S protein and thus appear to have been esterified under the conditions of this experiment.

One way that actual esterification of inorganic phosphate with ADP could be demonstrated would be to use $^{32}PO_4^{\equiv}$ in the medium and isolate pure ATP. If the isolated ATP contained ^{32}P activity this would be irrefutable evidence that the Fe-S protein catalyzed the esterification of inorganic phosphate into a high energy configuration. The total cpm of ^{32}P in ATP isolated from an incubation mixture

TABLE 9

ESTERIFICATION OF INORGANIC PHOSPHATE IN THE PRESENCE OF THE Fe-S PROTEIN

System	Picomoles of P[1] Esterified
Complete[2]	600[3]
Fe-S Protein, 20 mg	100
Without added ADP	200
Without added Fe-S protein	0
With 2 ml TCA added	0

[1]Corrected for the phosphate in ADP.

[2]40 mg Fe-S protein, 20 μmoles ADP, 20 μmoles of phosphate, 1 ml 0.1 M imidazole buffer, pH 7.4.

[3]Averages of duplicate determinations of a model experiment.

containing Fe-S protein, $^{32}PO_4^{\equiv}$ and ADP are shown in Table 10. These data show that Fe-S protein catalyzes the esterification of inorganic phosphate into the high energy configuration of ATP, and that the amount of esterification is proportional to the time of incubation. Paper chromatography demonstrated that the radioactivity moved with a known marker spot of ATP.

TABLE 10

EFFECT OF Fe-S PROTEIN ON THE INCORPORATION OF ^{32}P INTO ATP

System	Total ^{32}P Activity in Isolated ATP
	cpm x 10^{-4}
Complete[1]	
Incubated 20 minutes	0.6[2]
Incubated 45 minutes	2.6
Incubated 90 minutes	3.6
With added $MgCl_2$, 200 μmoles	1.0
Without added Fe-S protein	0.9

[1]40 mg Fe-S protein, 20 μmoles ADP, 20 μmoles of phosphate, ^{32}P (5.5 x 10^5 cpm), 1.0 ml 0.1 M imidazole buffer, pH 7.4. All systems except those indicated were incubated for 45 minutes.

[2]Averages of duplicate determinations.

CHAPTER V

DISCUSSION

Data have been presented (Table 2, page 34) to show that a sulfur-containing protein (S-protein) observed previously (1) to be unaffected by avitaminosis E or the stress of malathion intoxication (2) was also unaffected by the level of inorganic sulfate in the diet. Previous investigations (1,2,53,60,61) have shown that cellular lipoproteins, mucopolysaccharides, sulfolipids, bone epiphysis, taurine excretion, the glycocholic:taurocholic acid ratio and feed efficiency were affected by one or more of these conditions. These data serve to stress the importance of the S-protein and indicate that the uptake and release of sulfate by the S-protein is a high priority reaction for the integrity of the cell.

Because the S-protein had the most rapid turnover of any of the sulfur compounds investigated (1) including nucleotide-sulfur, the fraction which contained phosphoadenosine phosphosulfate (PAPS), it was assumed that the S-protein served as an intermediate in the sulfation of those cellular constituents which contained ester sulfate. Investigations designed to confirm the intermediary role of S-protein in sulfate esterification reactions were somewhat confusing. When a natural sulfate acceptor, mucopolysaccharide, was used in the test the S-protein appeared to possess the energy required to raise inorganic sulfate to the level of an ester sulfate (Table 4, page 36). However, when an artificial sulfate acceptor, p-nitrophenol, was used the S-

protein was found to possess little sulfation potential. It is conceivable that the sulfotransferase preparation used in these experiments was not free of sulfurylase and that any p-nitrophenyl sulfate formed was immediately hydrolyzed. Therefore, the measured result would be little sulfation of p-nitrophenol.

If the sulfotransferase were contaminated with sulfurylase, it was a fortunate error, because the experiments with the mucopolysaccharides had suggested that the S-protein possessed a high energy configuration and those with p-nitrophenol forced additional research into the role of S-protein in the cell. Since the above data indicated that the S-protein possessed a high energy configuration, the remaining experiments were designed to determine the relationship of the high energy configuration to the oxidative reactions of the cell. Conservation of energy by the formation of thioesters is not a unique process in cellular metabolism; for example, the energy of the oxidation of glyceraldehyde-3-phosphate is conserved by the formation of a thioester. However, the sulfur in glyceraldehyde-3-phosphate dehydrogenase is present as a sulfhydryl group. Since the sulfate exchange demonstrated in this investigation involves inorganic sulfate it is unlikely that the high energy configuration of the S-protein was generated during substrate level phosphorylation. As stated previously Fe-S proteins have been implicated in both electron transport and the energy conservation reactions of Complex I of the respiratory chain (18). Since the S-protein was demonstrated to contain iron it seemed reasonable to assume that the S-protein might be the Fe-S protein in which the energy of oxidation

was conserved at Site I. This assumption was reinforced by the demonstration of a modified sulfhydryl-disulfide exchange reaction between the Fe-S protein and 2-mercaptoethanol, and the suggestion by Gutman et al. (59) that a sulfhydryl $\longrightarrow$ disulfide oxidoreduction may participate in the conservation of energy at Site I.

As stated earlier (36) and shown in Figure 4, page 15, three Fe-S proteins have been implicated as part of the NADH dehydrogenase complex, some of which may be involved in actual electron transport. However, investigations in which iron was limiting in the growth media for yeasts have shown that electron transport but not energy conservation can occur when some of these Fe-S proteins are limiting (12,24-27). Thus it appears that some of the Fe-S proteins of the NADH dehydrogenase complex are involved in energy conservation. The Fe-S protein investigated in this study appears to be one of those Fe-S proteins involved in energy conservation. When the Fe-S protein was isolated from rats fed 2,4 dinitrophenol there was an increase in its specific activity. The increase in the specific activity of the Fe-S protein by rats fed 2,4 dinitrophenol was demonstrated to reflect an increased de novo synthesis of Fe-S protein by the demonstration of equal molar uptake of $^{59}Fe^{++}$ and $^{35}SO_4^{=}$ (Table 8, page 41). The increased synthesis of Fe-S protein may result from an attempt by the animal to compensate for decreased energy conservation. The latter supposition suggests that 2,4 dinitrophenol interferes with the sulfate-phosphate transfer reaction of the Fe-S protein.

The rationale for suggesting a sulfate-phosphate transfer reaction for the Fe-S protein comes from those experiments with the

esterification of inorganic phosphate (Table 9, page 43), the formation of radioactive ATP from ADP and $^{32}PO_4^{\equiv}$ (Table 10, page 45) and those experiments with the esterification of sulfate (Tables 3 and 4, pages 34 and 36). The first two experiments prove that the Fe-S protein can catalyze the esterification of ADP to form ATP. However, the experiments with the esterification of inorganic sulfate proved that the Fe-S protein could also provide the energy for the sulfation of ester sulfates, and that the sulfate was attached to the Fe-S protein before transfer to the mucopolysaccharides. These data (Tables 3 and 4) also demonstrated that ATP, the product of the reaction documented by the data presented in Tables 9 and 10, was inhibitory to the esterification of sulfate. A phosphate buffer was used in those experiments in which the esterification of sulfate was measured.

As shown in Figure 6, consideration of the above data allows the following proposal for the role of Fe-S protein in the cell. The Fe-S protein is part of the NADH dehydrogenase complex which traps the energy of electron transport by oxidatively binding inorganic sulfate to a sulfydryl group of the Fe-S protein, in a high energy configuration. A requirement for an intact sulfhydryl group for energy conservation at Site I has been demonstrated (59). Fe-S protein~S then picks up inorganic phosphate forming a high energy Fe-S protein~P and releasing sulfate. If a sulfate acceptor is available the released sulfate may be esterified; however, due to the particulate nature of the Fe-S protein, its cellular role as an intermediate in sulfation may be limited. Rather in the cell Fe-S protein~P reacts with ADP to generate ATP and regenerate the Fe-S protein.

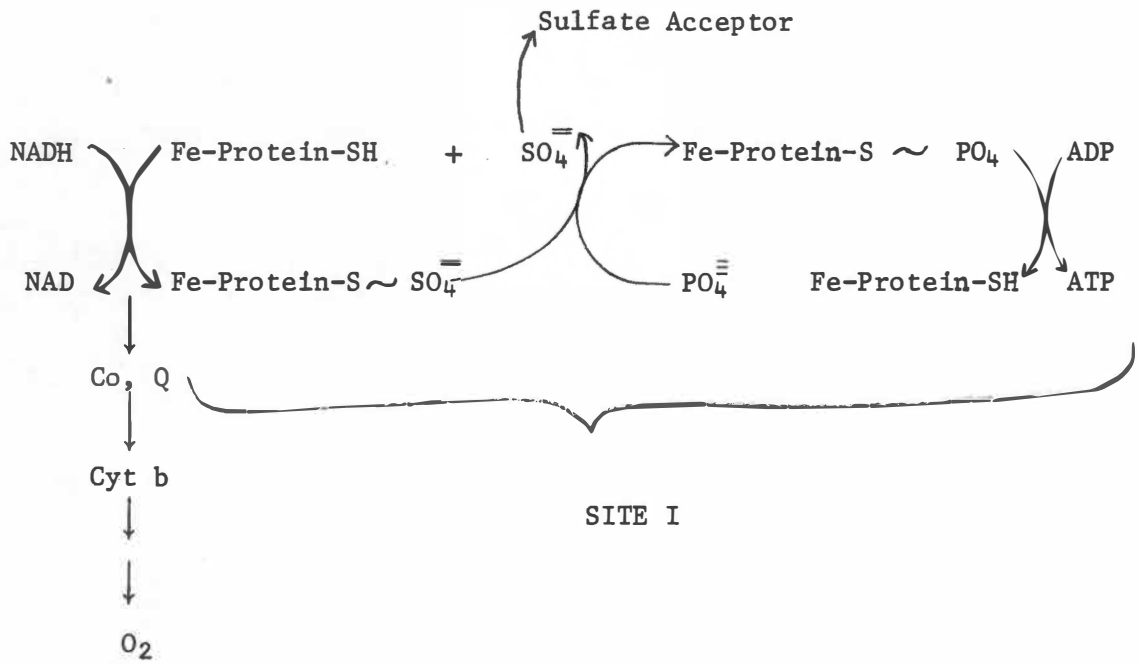

Figure 6. Scheme for the proposed role of the Fe-S protein in energy conservation at Site I.

CHAPTER VI

SUMMARY

The role of a sulfur-containing protein (S-protein) shown to have a high sulfate priority in the metabolism of the cell was investigated with respect to the formation of ester sulfates. When mucopolysaccharide was used as a sulfate acceptor the S-protein was shown to possess the energy required to raise inorganic sulfate to the energy level of ester sulfate. However, when the artificial sulfate acceptor, p-nitrophenol, was used as a sulfate acceptor no esterification of sulfate was observed.

These data, together with a consideration of the particulate nature of the S-protein and the demonstration that it contained iron, suggested that the S-protein (Fe-S protein) might be a component of the respiratory chain. This supposition was strengthened by the demonstration that 2,4 dinitrophenol resulted in an increase in the $^{35}SO_4^{=}$ specific activity of the Fe-S protein and also by the incorporation of equal molar amounts of $^{59}Fe^{++}$ and $^{35}SO_4^{=}$ into the protein. These data indicated that the animal was synthesizing extra Fe-S protein to compensate for the uncoupling reagent and the decreased energy conservation.

The high energy configuration of the Fe-S protein was proven by demonstrating that it would catalyze the esterification of inorganic phosphate with ADP as an acceptor, and the formation of radioactive ATP with $H_3{}^{32}PO_4$ as the donor and ADP as the acceptor.

Therefore, it is concluded that the Fe-S protein is a component of the NADH dehydrogenase complex and that it functions to trap a portion of the energy of the respiratory chain by oxidatively binding inorganic sulfate, which it could then exchange for inorganic phosphate. The high energy phosphate could then be transferred to ADP to generate ATP.

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VITA

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