NONHEME IRON AS A COFACTOR IN RIBONUCLEOTIDE REDUCTASE FROM E. COLI[1)]

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Ribonucleoside diphosphate reductase from Escherichia coli catalyzes the following reaction (Holmgren et al., 1965):

Ribonucleoside 5'-diphosphate + thioredoxin-$(SH)_2$ ⟶ Deoxyribonucleoside diphosphate + thioredoxin-S_2

Thioredoxin, the hydrogen donor in this reaction, is a small protein containing two half cystine residues which can exist either in a reduced (thioredoxin-$(SH)_2$) or oxidized (thioredoxin-S_2) state (Laurent et al., 1964). The reductase consists of two nonidentical subunits (proteins B1 and B2) which may be purified independently and recombined in the presence of Mg^{2+} to form the active enzyme complex (Brown et al., 1967). Separately each subunit is completely inactive. Protein B1 appears to have a regulatory function. The function of protein B2 has not been characterized.

During the reduction of ribonucleotides the 2'-hydroxyl group is stereospecifically replaced by hydrogen - probably in the form of a

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hydride ion (Larsson, 1965; Durham et al., 1967). It is difficult to visualize the direct generation of a hydride ion from the dithiol of thioredoxin without the participation of an intermediate hydrogen carrier. In the ribonucleoside triphosphate reductase system from L. leichmannii cobamide coenzyme apparently can function as such an intermediate (Blakley and Barker, 1964; Abeles and Beck, 1967). In the E. coli system, however, cobamide coenzyme is not involved.

In this paper we present evidence for the presence of bound iron as an integral part of protein B2. We suggest that iron is intimately involved in the reaction mechanism and that its function in the E. coli reductase may be similar to that of cobamide coenzyme in the L. leichmannii system.

Experimental

Enzyme purification - Proteins B1 and B2 were purified essentially as described earlier (Holmgren et al., 1965). The starting material was derived from E. coli B3 derepressed by growth on limiting amounts of thymine. For protein B2 the final purification procedure involved chromatography on hydroxylapatite followed by chromatography on DEAE-cellulose with a gradient of phosphate buffer (0.1-0.35 M, pH 6.3). The latter step was used in place of the previously described procedure involving chromatography on TEAE-cellulose with EDTA and phosphate buffer at pH 7.0. The details of the modified procedure will be published elsewhere.

Protein determination - The absorbance at 280 mμ was used as a measure of protein concentration. A provisional value of 1.3 (1 cm light path) is assumed for the absorbance of protein B2 at a concentration of 1 mg of protein per ml. This value was calculated from refraction data obtained in the ultracentrifuge by using a synthetic boundary cell and interference optics.

Iron and sulfur determination - Nonheme iron was determined by the o-phenanthroline method described by Massey (1957). Total iron after combustion with acid and labile inorganic sulfide were determined as described by Kimura and Suzuki (1967). In one experiment sulfide analysis was performed at 80° to ensure that all labile sulfide was dissociated from the protein (Lovenberg and Sobel, 1965).

Results and Discussion

General properties of protein B2 - After purification on DEAE-cellulose, protein B2 moved essentially as a single protein species during chromatography on Sephadex G-200 and displayed a constant specific activity in the peak fractions collected. However, the product after Sephadex chromatography was not completely pure, since data from sedimentation equilibrium centrifugation revealed a slight upward curvature in the plot of ln c against x^2. The average molecular weight for protein B2 obtained from this centrifugation was 82,000 with extreme values of 78,000 at the meniscus and 87,000 at the bottom of the cell. These values were calculated with an assumed partial specific volume of 0.74 ml per g of protein. One mg of protein B2 formed 0.8-1.2 μmoles of dCDP per minute at 25° under standard conditions in the presence of an excess of protein B1.

Spectrum and iron content of protein B2 - A spectrum of protein B2 after DEAE chromatography possessed a sharp peak at 410-411 mμ and a broader peak around 360 mμ (Curve 1, Fig. 1). When the protein was dialyzed for 63 hours at 4° against a solution of 0.05 M Tris buffer (pH 7.6) saturated with 8-hydroxyquinoline both peaks disappeared (Curve 2) and enzyme activity was lost completely. Addition of a small amount of $Fe(NH_4)_2(SO_4)_2$ (50 mμmoles per mg of protein), followed by dialysis against 0.05 M Tris buffer, pH 7.6, to remove excess iron, completely restored enzyme activity and resulted in the reappearance of the two peaks around 360 and 410 mμ (Curve 3).

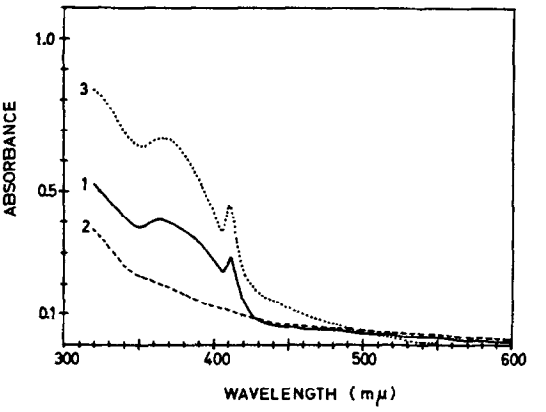

Fig. 1. Spectra of protein B2. Curve 1: enzyme after DEAE chromatography. Curve 2: enzyme after dialysis against 8-hydroxyquinoline (apoprotein). Curve 3: Apoprotein after treatment with iron. All curves are normalized to protein concentrations of 13 mg per ml.

Neutron activation analysis of protein B2 indicated the presence of considerable amounts of iron. No significant amounts of several other metals (Cu, Co, Mo, Zn, Cd, Cr) were found with this procedure. Quantitative analysis of the iron content was performed either with or without prior combustion of the protein. In both instances a value of 23 mμatoms of iron per mg of protein B2 (corresponding to curve 1) was found. The corresponding values for the proteins represented by curves 2 and 3 were 4 and 20 mμatoms, respectively.

This experiment demonstrated that the typical spectrum and the catalytic activity of protein B2 are linked to the presence of iron in the enzyme.

Additional evidence that iron forms an integral part of protein B2 comes from purification data. The amount of iron per mg of protein increased greatly during the preparation. After chromatography on hydroxylapatite the ratio of iron content to specific enzyme activity became constant. At the present stage of purification protein B2 contains 1.9 moles of iron per 82,000 g of protein.

These results clearly demonstrate that protein B2 contains iron as a tightly bound coenzyme. Moore and Hurlbert (1962) have already demonstrated the stimulation of a mammalian ribonucleotide reductase system by $FeCl_3$. However, the complexity and the low degree of purity of the mammalian ribonucleotide reductase system made it difficult to evaluate this finding in more detail.

During recent years numerous nonheme iron proteins have been described which participate as electron carriers in different biological systems (Malkin and Rabinowitz, 1967). Most of these proteins contain stoichiometric amounts of iron and inorganic sulfide. We could not demonstrate the presence of this type of sulfur in protein B2. The sharp peak at 410 mμ in the spectrum also serves to distinguish protein B2 from other known nonheme iron proteins.

Presently, the possible function of iron during the reduction of the ribose can be only a matter for speculation. The mechanism of the ribonucleoside diphosphate reductase appears to be best described as a replacement of the 2'-hydroxyl group by a hydride ion with the possible formation of a carbonium ion intermediate. It was suggested earlier that the enzyme from E. coli may contain a cofactor, analogous to the cobamide coenzyme of the L. leichmannii system, which is located in the electron transport chain between dihydrothioredoxin and ribose and functions in the generation of a hydride ion (Reichard, 1968). The results reported in this paper make it conceivable that nonheme iron is such a cofactor. It is also possible that iron may have an additional or alternative function in the catalytic mechanism such as that of an intermediate acceptor of the hydroxyl group removed during the enzyme reaction.

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