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Mutual Interactions between Macromolecular Reducing Substrates in *Pseudomonas aeruginosa* Nitrite Reductase

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The existence "*in vitro*" of more than one macromolecular electron donor (azurin and cytochrome c_{551}) for the enzyme which reduces NO_2^- to NO in the respiratory chain of *Pseudomonas aeruginosa* (E.C. 1.9.3.2.), poses a number of problems relevant to a more complete understanding of the bacterial respiratory pathways [1]. From the biochemical point of view a crucial question is to define to what extent the molecular mechanisms of electron transfer from the two substrates to the enzyme are similar. The problem was attacked by kinetic methods under pre steady-state and steady-state conditions, using either only one or both substrates simultaneously. The results of these studies indicate that: i) cytochrome c_{551} is a more rapid electron donor, compared with azurin, by a factor of 4–5 [2]; ii) the two substrates display a partially competitive inhibition.

Given the presence of a very fast electron transfer between azurin and cytochrome c_{551} [3], however, the latter result by itself does not permit the definition of the nature of the reactive site(s) on the enzyme. A more direct answer to the problem may be obtained using Hg-substituted azurin [4], which is ineffective as a redox substrate, but still reacts with the specific binding sites on the enzyme. This may allow a straightforward interpretation of the observed kinetic patterns in mechanistic terms.

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O33

Endor of Metalloenzymes

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Much of the current understanding of metalloenzyme structure and function has been obtained by application of spectroscopic methods, in particular, electron paramagnetic resonance (epr) techniques. Nevertheless, epr studies often are of limited utility because hyperfine interactions from constituent or substrate nuclei are unobservable. Electron-nuclear double resonance (endor) is a high-resolution extension of epr that can often resolve these interactions, thus removing the limitation. In endor, nuclear resonance transitions are observed through their influence on the strength of the epr signal and, unlike ordinary nmr, they can be comparably observable for all magnetic nuclei. Thus, the technique offers some unique capabilities for the characterization of an enzyme catalytic center. We will describe the application of endor to metalloenzyme resting states and reaction intermediates by choosing examples from systems we have studied, which include cytochrome oxidase, peroxidases, and nitrogenase; resonances from ^{1}H, ^{14}N, ^{17}O, ^{57}Fe, $^{63,65}Cu$, and ^{95}Mo may be discussed.

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Bacterial Cadmium-binding Proteins

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There is much current intererest in the metallothioneins. These are low molecular weight proteins (6–7,000 Daltons) which have a high cysteine (33%) and metal content (7 g atoms/mol). This, together with the absence of aromatic amino acids and histidine, makes this protein very unusual [1].

Metallothioneins have no defined functions, although many possible roles have been suggested including detoxification [2], and regulation of zinc [3] and copper metabolism [4].

Metallothioneins are widespread in mammals [1] and have also been isolated from fish [5], invertebrates [6], plants [7] and eukaryotic microorganismas such as yeast [8] and *Neurosporra crassa* [9].

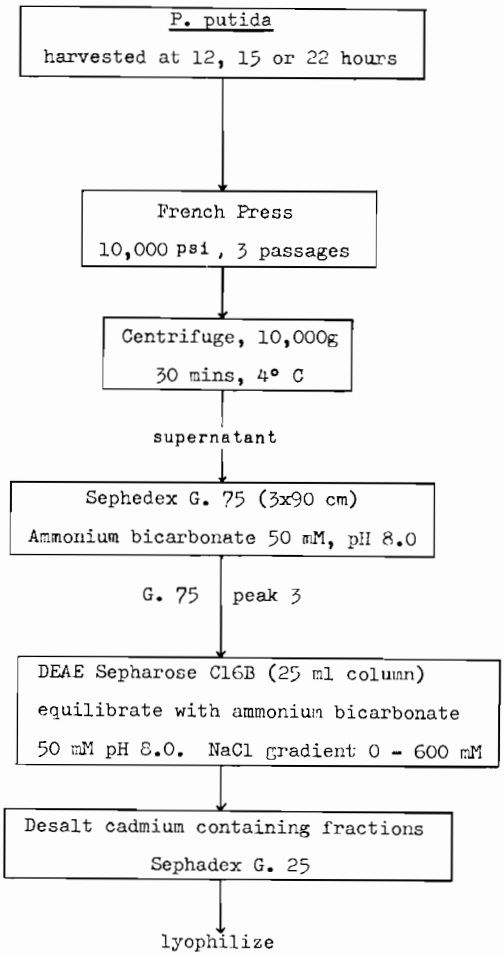

Fig. 1. Isolation of cadmium binding proteins for *P. putida.*

The *N. crassa* protein has been sequenced and found to have extensive homology with mammalian metallothionein [9].

Metallothioneins or similar proteins have never been isolated from bacteria. We have studied the mechanism of cadmium resistance in a gram negative bacterium and isolated novel cadmium binding proteins. The study of these bacterial proteins may provide new insight into the metabolism and probable function of metallothioneins.

Three different cadmium-binding-proteins are produced by a cadmium-resistant strain of *Pseudomonas putida,* isolated from sewage sludge. The bacterium accumulates cadmium from the environment, and the internal concentration can reach 9 m*M.* Up to 30% of this cadmium is found in the cytoplasm. Three cadmium binding proteins were isolated from the cytoplasm of *P. putida* (Fig. 1). Each protein is produced during a different phase of the growth cycle (Fig. 2).

The proteins have a high cysteine content and bind cadmium, copper and zinc. They are induced

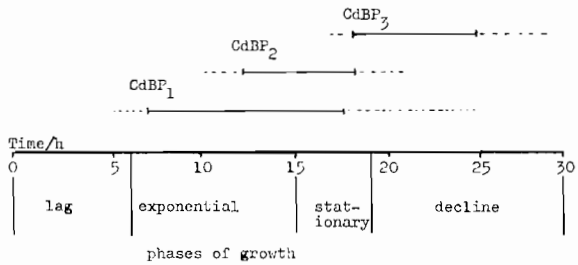

Fig. 2. Production of cadmium-binding proteins $CdBP_1$, $CdBP_2$ and $CdBP_3$ at various stages of the growth cycle of *P. putida.*

by the presence of cadmium. Cadmium-binding-protein 1, molecular weight 7,000 ($CdBP_1$) binds 7 g atoms metal/mol, and $CdBP_2$ (7,000 daltons) binds 3 g atoms metal/mol. $CdBP_3$, is a much smaller molecule (3,800 daltons) and binds cadmium and copper (4.5 g atoms metal/mol).

All three cadmium-binding proteins contain large amounts of cysteine, but only about half that commonly found in metallothioneins. $CdBP_3$ has the highest cysteine content of 22%.

Also typical of the metallothioneins is the high serine, glycine, and alanine contents of $CDBP_1$ and $CdBP_2$. $CadBP_2$ also contains substantial amounts of lysine. Both $CdBP_2$ and $CdBP_3$ have few or no residues of arginine, leucine, isoleucine, or the aromatic amino acids.

The major differences from metallothionein are the high glutamate content of $CdBP_2$ and $CdBP_3$. $CdBP_1$ also contains substantial amounts of leucine, isoleucine and also four residues of aromatic amino acids.

^1H NMR experiments at 400 MHz support the amino acid analysis, revealing the lack of aromatic amino acids in $CdBP_2$ and $CdBP_3$ as well as the high cysteine content.

The high metal-binding capacity of these proteins, but the lower content of cysteine, suggests that in addition to cysteine-SH other protein ligands may be involved in cadmium binding.

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X-Ray Absorption Spectroscopy of 3-Fe Clusters in Fe–S Proteins

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Fe EXAFS and edge spectroscopy have been used to characterize the 3-Fe clusters in aconitase and *Azotobacter vinelandii* ferredoxin I (*Av* Fd I). Fe EXAFS of a frozen solution of oxidized (unactivated) beef heart aconitase indicates a 'compact' cluster structure with Fe–Fe distances of *ca.* 2.7 Å. In combination with independent iron and acid-labile sulfur determinations, these results allow us to propose a structure for the aconitase 3-Fe cluster with a stoichiometry of [3Fe-4S].

Single crystal polarized X-ray absorption spectroscopy was performed on *Av* Fd I crystals with the goal of distinguishing Fe–Fe scattering contributions from the 4- and 3-Fe clusters. Crystallographic results on this protein suggest an 'extended' structure for the 3-Fe cluster with *ca.* 4.1 Å Fe–Fe distances. Polarized spectra were recorded with the X-ray polarization vector both normal to and parallel to the average plane of the irons in the 3-Fe clusters. The Fourier transforms were compared and found to be identical in the region in which a 4.1 Å scattering peak would be expected. Thus, the long (*ca.* 4.1 Å) Fe–Fe distance cannot be detected in *Av* Fd I crystals at room temperature. Low-temperature solution work is planned to look for evidence of this interaction.

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A Water Proton and Anion Affinity Investigation of Zinc(II) Deprived Superoxide Dismutase

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In native superoxide dismutase, copper(II) is bound to four histidine nitrogens in a puckered square planar arrangement, the angles N–Cu–N for the two couples of trans nitrogen atoms being 160° and 230°, respectively. One of these nitrogens belongs to an histidinate residue bridging the zinc ion [1]. The EPR spectrum shows a large rhombic component [2]. Anions like CN^-, N_3^-, NCS^-, NCO^- substitute in our opinion for a histidine nitrogen [3, 4].

Zinc deprived SOD shows an axially symmetrical EPR spectrum [5] indicating that the removal of the bridging requirement leads to a less distorted chromophore. Water proton NMRD of solutions containing SOD have indicated the presence of a coordinated axial water molecule [6]. The same measurements performed on the zinc deprived derivative (Fig. 1) have shown that water is still present, although loosely bound as in the native SOD.

The affinity constants of the anions N_3^- and NCS^- at pH 6.0 for zinc deprived SOD (160, 26 M^{-1}) closely compare with those for the native enzyme (90,

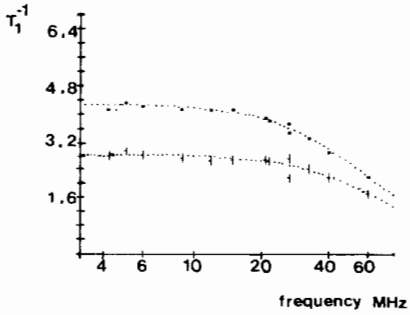

Fig. 1. Magnetic field dependence of the proton relaxation rate of aqueous solutions of native (▪) and zinc deprived (+) SOD. The samples were 5.6×10^{-4} M protein, pH 6.0.