The evolutionary choice of Ca^{2+} as a biological messenger has in all likelyhood been dictated by its chemical properties, which greatly favor it over the other cations available in the original environment (K^+, Na^+, Mg^{2+}, NH_4^+). The divalent ion Ca^{2+}, which has a radius of slightly less than 1 Å, is ideally qualified to fit irregular binding cavities of the type normally encountered in folded proteins. A comparison with the other divalent cation originally available, Mg^{2+}, (ionic radius 0.65 Å) offers a plausible explanation for this. The smaller polarising power of Ca^{2+} imposes no stringent geometric demands on the cavity designed to accept it, whereas the greater polarizing power of Mg^{2+} does. The flexibility in accepting binding cavities is evidently a decisive advantage for an ion designed to act as a messenger. This is so because the messenger function requires that the ion be kept at very low activity levels inside cells, where the targets of the messenger function are found. This evidently requires easy binding of the ion to complex X_L (protein) ligands of the type found inside cells. The messenger function also requires that the activity of the messenger ion inside cells be rapidly and precisely modulated around the low background level, and this in turn demands that the interaction of the ion with the complex ligands be easily reversible. Soluble and membrane-bound ligands are both convenient, the latter having the additional advantage of modulating the activity of Ca^{2+} into cells by mediating its reversible transport across membrane phases into different aqueous compartments.

The importance of the messenger function of Ca^{2+} is underscored by the existence of a large number of intracellular specific binding proteins, and by the fact that a variety (up to 7) of systems exist for its transport across cellular membranes. The redundancy of the transport systems is a particularly striking reminder of the absolute necessity of controlling the levels of free Ca^{2+} in all compartments of the cell. The fact that completely different transport modes often coexist in the same membrane is evidently dictated by the necessity of controlling Ca^{2+} with precision and flexibility. This is so because the various transport systems have characteristics (K_m, V_{max}, specificity, inhibition, regulation) that qualify them ideally for intervening at specified moments of the physiological life of cells.

So far, the following Ca^{2+} transport modes have been described in different membranes: 1) 2 specific pumping ATPases, 2) 2 Ca^{2+}/Na^+ exchange systems, 3) at least one Ca^{2+}/H^+ exchange system, 4) one electrophoretic, charge uncompensated transport system. Some of these transport systems have been solubilized, purified, reconstituted, and are now amenable to molecular studies. Others can at the moment only be studied functionally, in situ. Of particular interest is the finding that calmodulin, perhaps the most interesting of all intracellular Ca^{2+} binding proteins, and undoubtedly the most important carrier of the Ca^{2+} message to (enzyme) targets, stimulates some of the systems for the transport of Ca^{2+} across membranes. A comprehensive picture would thus have Ca^{2+} not only regulating scores of important enzymic activities within cells, but even its own balance between cells and the environment, and among the various compartments of the cell.

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Deletion Mapping of the Aerobactin Gene Complex of Plasmid ColV

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With the possible exception of certain strains of lactic acid bacteria, iron is known to be an essential element for all species of microbes and for all higher organisms. Within the cell, iron protein catalysts participate in some of the reactions most fundamental to life, including aerobic and anaerobic energy metabolism, nitrogen fixation, photosynthesis, and generation of the deoxyribotides required for synthesis of DNA. Iron is abundant in the environment but may be insoluble or in some other way unavailable. Mechanisms for assimilation of iron have been evolved by all species but, because of the plasticity and diversity of microbial metabolism, those in unicellular organisms are the most readily studied.

The particular system of microbial iron acquisition investigated in this laboratory is labelled *high-affinity* and consists of low molecular weight, virtually Fe(III) specific ligands, termed siderophores, and the matching membrane receptors for the complexed Fe(III) ion. This system, which is coordinately expressed under iron starvation, has been detected in virtually all aerobic and facultative anaerobic microorganisms carefully examined for its presence [1].

In general, siderophores can be relegated to one of two chemical classes, *viz.*, hydroxamic acids and catechols. The number of siderophores characterized to date, some by crystallography and/or high resolution nuclear magnetic resonance spectroscopy, must now number several score. The complete system, comprised of siderophore and specific receptor, is

most effectively investigated in the enteric bacterium *Escherichia coli*, an organism for which detailed genetic information is available. Enteric bacteria commonly form a chromosomally encoded catechol-type siderophore, enterobactin, and an 81.000 (81K) dalton outer membrane receptor for ferric enterobactin. Membrane receptors for ferric siderophores are known to serve as sites of attachment or penetration of lethal agents such as certain specific bacteriophages, bacteriocins and antibiotics [2].

Since iron is toxic in excess and since there is no known biological mechanism for its excretion, internal iron is shuttled between stores and the physiological sites of action. The uptake of new iron is regulated precisely at the membrane level.

We have sought to understand the molecular mechanics of regulation of siderophore mediated iron assimilation by investigation of the aerobactin system encoded on the ColV-K30 plasmid of *E. coli.* The presence of this system on a plasmid, albeit a large one (90 kb), greatly facilitates subsequent genetic manipulations. Aerobactin, as a hydroxamic acid, is representative of a type of siderophore which occurs in both prokaryotic and eukaryotic worlds while the catechol, enterobactin, is a siderophore apparently confined to enteric bacteria. A relatively simple system should be required for biosynthesis of ferric aerobactin, and for the regulation of this process. Aerobactin has the added interest of being the siderophore which correlates with virulence in clinical isolates of *E. coli.* This is the philosophy and the rationale for our focus on the molecular genetics of the aerobactin system.

Using a selection based on sensitivity to cloacin, the aerobactin gene cluster of plasmid ColV-K30 was cloned on pPlac, a small multi-copy vector bearing ampicillin resistance [3]. The derived plasmid, 18.3 kilobases (kb), was designated pABN1 and shown to encode the entire gene sequence for aerobactin synthesis and the outer membrane receptor. A subclone, pABN5, 8.7 kb, lacked the gene for the ferric aerobactin/cloacin 74K outer membrane receptor.

In order to determine the number, relative location and function of the genes in the aerobactin complex, we have performed a systematic deletion of various restriction fragments of pABN1. This paper constitutes a preliminary account of our findings.

Experimental

Using the information contained in the detailed restriction maps [3] for plasmids pABN1 and pABN5, four deletion plasmids were constructed and designated pABN7, 11, 13 and 15. Mini-cells of *E. coli* X1488 containing the plasmids were charged with ^{35}S-methionine and the proteins synthesized then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography in the usual way [4].

Results and Discussion

Mini-cells containing the largest plasmid, the 18.3 kb pABN1, were observed to form five proteins, in addition to the 30 K β-lactamase from the vector, with molecular weights of 27K, 33K, 53K, 63K and 74K. Protein 74K was expressed by pABN1 and 7. Protein 63K was expressed in all deletions and its gene must be confined to a 2.1 kb HindIII-AvaI fragment immediately adjacent to the vector and must be preceded by a promoter. Component 53K was formed by all deletions except pABN13 and 15, while 33K appeared in every deletion except pABN15. In pABN11 the 27K band appeared at a slightly lower position on the gels. Because of background from the vector, this region of the gels may require further analysis.

The pattern of expression of the five proteins coupled with knowledge of the location of the deleted sequences of DNA in the eight plasmids suggests the gene order 63K, (27K + 33K), 53K, 74K.

From previous work [5] and from phenotypic sensitivity/resistance to the bacteriocin cloacin we know that 74K is the outer membrane receptor for ferric aerobactin. The 27K protein may be the synthetase, or a portion thereof, since cells with pABN11 formed a bioinactive hydroxamate, possibly the side chain of aerobactin. We do not yet know the biofunction of the other three proteins, which are not correlated with formation of any ferric perchlorate positive material. Biosynthesis of aerobactin would require, in addition to a synthetase, a lysine oxygenase and an acetylase.

Our results are most readily explained by assuming that the aerobactin gene complex is contained within a single operon which is expressed from left to right in the restriction map of pABN1 given earlier [3]. The relatively inefficient regulation by iron reported [3] in the cloned sequences may be assigned to the high copy number of the recombinant plasmids and to the presence of a regulatory system consisting of a chromosomally encoded repressor protein which binds iron as a co-repressor. This form of regulation, which has been assumed for the past decade [6], has yet to receive direct experimental verification.

Work in progress on the molecular genetics of the aerobactin system may afford the first detailed description of the organization, structure and regulation of a hydroxamate type siderophore operon, which we estimate to be some 7 kb in size. The chromosomally coded ferric enterobactin system of *E. coli* has been cloned on phage Mμ and shown to be organized into at least six transcriptional units extending over about 26 kb of DNA [7].

Experiments in this laboratory [8] on the equilibration of aerobactin and enterobactin with ferric transferrin indicate that in buffer the latter siderophore removes iron at a superior rate while in serum

the converse is true, possibly because of the aromatic character of enterobactin and its propensity to adhere to proteins such as albumin.

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J5

The Two-Sited Nature of Transferrin: Spectroscopic, Thermodynamic and Physiologic Studies

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The transferrins comprise a class of two-sited metal-binding proteins widely distributed in cells and fluids of vertebrates. Each of the major transferrin varieties—serum transferrin, ovotransferrin (conalbumin) and lactoferrin (lactotransferrin)—consists of a single polypeptide chain with extensive internal homology indicating evolutionary origin from a single-sited precursor protein. In human transferrin and hen ovotransferrin, at least, evolutionary development has altered the two sites so that they are chemically and spectroscopically distinguishable, but whether the differences between the sites are physiologically important is not yet clear.

The metal-binding activities of all transferrins are dependent on the concomitant binding of structurally-suitable anions. In human serum transferrin, anion- and metal-binding activities alike vary between the two sites. For example, the sites may be distinguished by the thightness with which they bind ion, by their accessibility to different chemical forms of iron, by their vulnerability to acid, by the rates at which they exchange anions with the medium, by their capacity to accommodate different anions when Cu(II) is the bound metal ion, and by their spectroscopic features. When single-sited transferrin fragments are prepared by selective proteolytic digestion, the tightness of ion-binding by these fragments is virtually identical to that of corresponding sites in the holoprotein. Evidently, therefore, site-site interactions are not responsible for the observed differences between the sites in native transferrin.

Because each of the sites of transferrin resides in its own independent domain, and empty-sited domains are more susceptible to denaturation than occupied domains, each of the four possible molecular species of transferrin (apoprotein, monoferric-A and monoferric-B proteins, and differic protein) can be separated and displayed by electrophoresis in a 6M urea-gel system introduced by Makey and Seal. This procedure has been exploited to study the physiological iron-exchanging properties of the sites, *in vitro* and *in vivo*. In studies conducted in our laboratory, the two sites of diferric transferrin differ considerably in their ability to donate iron to isolated hemoglobin-synthesizing reticulocytes in an artificial culture medium. In *in vivo* studies in the rabbit, however, the two iron atoms are removed from diferric transferrin in pairwise fashion, so that the sites now appear equivalent in their iron-donating properties. Furthermore, iron, from stores and sites where it is absorbed or recovered from senescent red blood cells, is returned to empty sites of circulating transferrin, predominantly one atom at a time. Since studies of iron distribution among the sites of circulating transferrin in normal human subjects indicate that the sites are unequally occupied, with the more weakly binding site of the N-terminal half of the molecule favored, it seemed that the sites might differ not in their iron-donating abilities, but in their iron-accepting properties. Accordingly, the distribution of absorbed radioactive iron between the two sites of circulating transferrin was studied in the rabbit. In four of five animals the B-site was strongly favored by the newly absorbed iron. This may account, then, for the unequal distribution of iron between the sites of circulating transferrin. If the chemical differences between the two sites of transferrin are mapped into differences in function, therefore, it is probably on the 'supply side' of the iron economy.

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