

Interaction and Compartmentalization of the Components of Bacterial Enzyme Systems Involved in Cell Energetics

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Z. Naturforsch. **48c**, 535–541 (1993); received May 25, 1993

Bacterial Enzymes, Cell Energetics, Enzyme Interaction, Compartmentalization

Bacterial enzyme systems, especially those which are involved in cell energetics, often show a common characteristic feature: their constituents (either interacting enzymes or subunits of a given enzyme complex) are physically separated. They are located in different functional entities, such as cytoplasm or periplasmic space. This kind of cellular and macromolecular organization enables the cell to establish spatially separated but neighbouring zones in which distinct conditions are created or maintained. This intrinsic imbalance is one of the keys for the process of life. As the mediator between the two compartments, the cytoplasm and the periplasmic space, the cytoplasmic membrane – itself a functional entity – not only acts as a barrier, but carries a set of functional enzyme components, thus contributing to the interaction between compartments. Examples to illustrate this concept are enzyme systems involved in anaerobic glycine metabolism, aerobic utilization of carbon monoxide, proton or sodium translocation across the membrane, and intracellular hydrogen cycling used by the cell for the generation of a proton gradient.

Introduction

Compartmentalization is one of the most obvious features of the cells of the eukaryotic organisms. Discrete cellular compartments, enclosed by membranes, fulfil specific and specialized roles [1], and their synthesis, maturation, activity and breakdown are regulated processes. These compartments are integrated into cellular metabolism *via* communication and interaction either across the membranes by which they are enclosed through transport processes, or by membrane fusion events. By contrast with eukaryotes, the structural and functional organization of the bacterial cell is much less obvious complex. Besides the cytoplasm proper, there are generally no closed compartments defined by membrane layers. The entire bacterial cell is generally thought of as a single compartment, comparable with the chloroplasts and the mitochondria of eukaryotic cells [2, 3]. Nevertheless, bacteria grow and divide, and are certainly no less sophisticated than eukaryotic cells in terms of overall metabolic activities. This appar-

ent paradox can be explained if bacteria have achieved the same end (effective compartmentalization of many cellular processes) by different means, and require only a single subdivision into two membrane-defined true compartments, the cytoplasm and periplasm.

With our understanding of the bioenergetics of the bacterial cell, the central role of the cytoplasmic membrane as both the mediator and interface between the cytoplasm and periplasm has been recognized. The cytoplasmic membrane functions both as a barrier and as the site of location of many proteins, a number of which are involved in regulated transport across the membrane. Additionally, many of these membrane-associated and membrane-bound proteins interact with proteins located in the adjacent cytoplasm or periplasm, and the energized state of the membrane which results from an imbalance established or maintained between these two compartments is one of the most basic features of living things. The present article uses examples from cellular energetics to illustrate the central role of the cytoplasmic membrane in the functional organization of the bacterial cell, and the usefulness of electron microscopic techniques for the evaluation of hypotheses concerning cellular energy conservation.

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Verlag der Zeitschrift für Naturforschung,
D-72072 Tübingen
0939–5075/93/0700–0535 \$01.30/0

Bacterial Enzyme Systems

Anaerobic glycine metabolism: an example of a shuttle protein mediating electron flow between enzymes

The key enzymes of glycine fermentation are a cytoplasmic glycine decarboxylase and a glycine reductase which is located in the cytoplasmic membrane [4].

As in other organisms, the glycine decarboxylase complex from *Eubacterium acidaminophilum* consists of four types of subunit (P1–P4). The P1 component catalyzes the decarboxylation of glycine, and the resulting amino-methyl group is attached to the lipoyl moiety of a second component, protein P2. This group is then cleaved to ammonia and formaldehyde or methylene tetrahydrofolate by the action of the transferase protein, P4. During decarboxylation, the lipoyl moiety of P2 is reduced; the reaction cycle is thought to be completed with the reoxidation of P2 and concomitant reduction of NAD(P)⁺ by the P3 component of the system, a lipoamide dehydrogenase.

The reducing equivalents generated by this first enzyme complex, consisting of glycine decarboxylase, methylene-tetrahydrofolate dehydrogenase and formate dehydrogenase, are transferred to a selenoprotein known as PA. This protein is a component of the second enzyme complex mentioned above, the membrane-bound glycine reductase, which uses the reducing equivalents supplied by the first complex to bring about the reduction of glycine.

Because it could function both as a lipoamide dehydrogenase during glycine decarboxylation and as an electron carrier during glycine reduction, it was considered possible that the P3 protein provided a functional link between the two enzyme complexes. In order to obtain some information about this and other possible interactions between the two enzyme complexes, the cellular location of the P1–P3 and PA proteins was investigated. The rationale here is that even though P3 is known to be cytoplasmic, it is unlikely to be distributed uniformly. Rather, a heterogeneous distribution is to be expected, particularly if P3 interacts with membrane-bound components of the glycine reductase complex. The approach taken was use of polyclonal antibodies against P1, P2, P3 or PA in immunocytochemical probing of low-temperature embed-

ded bacteria, using ultrathin sections. As well as the application of routine single-labelling post-embedding techniques, a series of double-labelling experiments, involving combinations of antibodies against P1/P2, P1/P3, P2/P3 and P2/PA were undertaken, and the data were evaluated both qualitatively and semi-quantitatively.

Single-labelling experiments implied that only minor proportions of P1 and P2 were found to be associated with the cell membrane, whereas P3 was predominantly found at the cell periphery. Double-labelling experiments implied that P1 and P2 were closely associated in about 50% of all cases. Somewhat less common (but still accounting for a substantial proportion, about 30 to 35% of cases) was an association of P2 and P3. Pairs P1/P3 were only rarely observed. Protein PA was found to be distributed at the cell periphery as well as in the cytoplasm in cells grown on glycine. Those molecules of PA located at the cell periphery appeared always to be closely associated with label representing protein P3. These data indicate that, although they are basically cytoplasmic, the glycine decarboxylase proteins P1 and P2 may be weakly associated with the cytoplasmic membrane. This finding is consistent with the previous failure to isolate glycine decarboxylase complexes containing all four component proteins (P1–P4) from *E. acidaminophilum*. Surprisingly however, protein P3, assumed to be a component of the (functional) cytoplasmic glycine decarboxylase, was found to be membrane-associated. The association of P3 with PA, a constituent of the (membrane-bound) glycine reductase, implies that electron flow from glycine decarboxylase to glycine reductase occurs predominantly *via* the protein P3 (formally a component of glycine decarboxylase) to protein PA of glycine reductase, although P3 is not complexed with the other components of glycine decarboxylase. It should be mentioned that the involvement of a flavoprotein, like the lipoamide dehydrogenase P3, in glycine reduction had been postulated long ago. However, although P3 was found not to form a complex with the other components of glycine decarboxylase, it is strictly required for the glycine decarboxylase reaction as well as for glycine reduction. Another relevant point is that protein P2 appears to be present in 10-fold molar excess over P1 and P3. Taken together, these observations support the model for

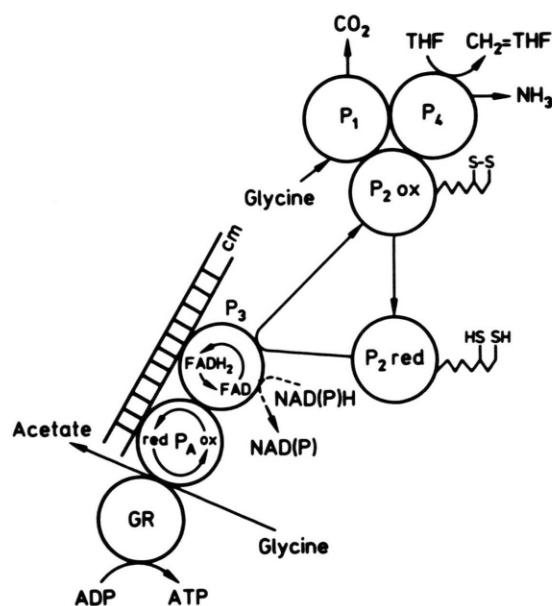


Fig. 1. Model for interactions of proteins of glycine decarboxylase with those of glycine reductase in *Eubacterium acidaminophilum*. P₁, pyridoxal phosphate-containing decarboxylase protein; P₂, lipoamide-containing hydrogen carrier protein; P₃, lipoamide dehydrogenase; P₄, transferase protein; P_A, selenoprotein; GR, glycine reductase; THF, tetrahydrofolate; CH₂=THF, methylene-tetrahydrofolate; cm, cytoplasmic membrane. (From ref. [4].)

glycine metabolism in *E. acidaminophilum* shown in Fig. 1. Lipoamide dehydrogenase protein P₃ and selenoprotein P_A, which are closely associated with each other and located in the vicinity of the cytoplasmic membrane, are central to coupling of glycine reductase to energy conservation in *E. acidaminophilum*. The protein complex consisting of P₁, P₂ and P₄ catalyzes a reaction leading to reduction of protein P₂; this protein is reoxidized by protein P₃ which is the only protein with lipoamide dehydrogenase activity in this bacterium. Because P₃ is not complexed with other components of glycine decarboxylase (including P₂), this implies that the reduced protein P₂ must dissociate temporarily from the glycine decarboxylase complex in order to transfer electrons to protein P₃ and thus become reoxidized. Thus P₂ may function as a hydrogen carrier between glycine decarboxylase and glycine reductase, by shuttling between the decarboxylase complex and lipoamide dehydrogenase (P₃).

Aerobic utilization of carbon monoxide: interaction of a facultatively cytoplasmic enzyme component with a membrane-integrated component of the enzyme

A number of phylogenetically-distinct bacteria have the ability to aerobically utilize carbon monoxide (CO) as sole source of carbon and energy. This process requires several biochemical components which are not present in other microorganisms. The key enzyme is CO dehydrogenase, and the best characterized example is from *Pseudomonas carboxydovorans* [5]. The CO₂ formed by the action of the CO dehydrogenase is subsequently assimilated via the reductive pentose phosphate cycle.

Surprising results were obtained when immunocytochemical techniques were applied to the localization of this enzyme [6]. Whereas during exponential growth the enzyme was attached to the inside of the cytoplasmic membrane, during stationary phase it was apparently predominantly cytoplasmic. The dissociation of the enzyme from the cytoplasmic membrane coincided with a decrease of CO-oxidation activity with O₂. It was shown that electron flow from CO to O₂ is controlled by the amount of CO dehydrogenase attached to a membrane-bound electron acceptor. This finding was supported by reconstitution experiments: *in vitro* reconstitution of depleted membranes with the corresponding supernatants containing CO dehydrogenase led to binding of the enzyme and to reactivation of respiratory activity with CO. Reconstitution worked with membranes from CO-grown bacteria, whereas cytoplasmic membranes from H₂- or heterotrophically grown cells did not bind CO dehydrogenase. Although the basis for the process of attachment/detachment of CO dehydrogenase is not yet understood, cytochrome *b*₅₆₁ has been unequivocally identified as the membrane-integrated part of the enzyme system.

In this example of a study on ultrastructural organization, systematic testing of various bacterial growth phases, guided by known physiological states of the cells, finally led to the detection of an interesting concept developed in the bacterial cell. It implies that several components of a given system, facultatively either located in the cytoplasm or attached to the cytoplasmic membrane, may be

coupled to one another, to perform a function which involves the interaction of compartments. In the absence of an appropriate, highly specific method for protein identification and localization, the dynamics inherent in the system would have escaped documentation at the ultrastructural level. In case of *P. carboxydovorans*, these studies in fact served as the basis for subsequent experiments at the physiological, biochemical and gene levels.

Proton or sodium translocation across the membrane: cooperation of membrane-integrated enzyme proteins with components of the enzyme system orientated towards the cytoplasm

Proton or sodium translocation across the cytoplasmic membrane is a process which determines the energetic state of the cell [7, 8]. These vital transport processes involve the cytoplasm, the periplasmic space (or the extracellular space), and the cytoplasmic membrane. Transport has to take place from a hydrophilic environment, through a hydrophobic "barrier", into another hydrophilic environment. Transfer through the membrane implies that within this barrier, functional "channels" or "pores" must exist to provide a connection between inside and outside of the cell. The constituents forming such "pores" may serve additional purposes: they may function as components of enzyme complexes to which other parts of the enzyme complex, *i.e.* those which are orientated towards the cytoplasm, are connected. Alternatively, such connections may be brought about by additional membrane-integrated proteins complexed with the "pore"-forming units.

A comparison of the typical FoF₁-ATPase with a sodium ion pumping system, such as oxaloacetate decarboxylase, shows similarities and differences. Structural similarities include a subdivision into a membrane-integrated part and a component exposed to the cytoplasm, the latter of which carries the catalytic site(s). Functional similarities are the involvement in ion translocation and cell energetics. In both cases, the "cytoplasmic" part of the enzyme catalyzes a reaction which is formally independent of ion translocation: free F₁ particles exhibit enzymatic activity as an ATPase, and the carboxyltransferase reaction catalyzed by the α subunit of oxaloacetate decarboxylase is independent of the presence of sodium ions. How-

ever, only interaction with the membrane-integrated components constitutes full functional activity.

From an ultrastructural point of view it is interesting to see how nature has organized such a complex enzyme system. The F₁ part of ATPase exhibits formal rotational symmetry caused by the oligomeric character of the complex [9]. Detailed studies by cryo electron microscopy of frozen-hydrated samples, combined with immuno electron microscopy, revealed function-dependent deviations from the ideal rotational symmetry. Systematic dislocations of the central components of the F₁ particle towards the subunits forming the peripheral enzyme mass (*i.e.* 3 α and 3 β subunits) appear to reflect specific functional states [10]. An investigation of structural details of the Fo part of *E. coli* ATPase which combines a dual function, *i.e.* formation of the functional "pore", and involvement in structural coupling of the F₁ and Fo parts has now been performed [11]. A former model [12] based on biochemical findings and the stoichiometry $a:b:c=1:2:(10-12)$ had postulated that the c subunits form a complex, that the a subunit is laterally attached to this complex, and that the two b subunits (which mediate the contact to the F₁ part) are laterally attached, at two opposite flanks, to the a/c complex at the sites of contact between the c complex with the a subunit (Fig. 2). Application of the negative staining technique combined with epitope mapping of the subunit types within the solubilized Fo complex (using monoclonal IgG antibodies), confirmed this model [11].

The electron microscopic visualization of Fo, with and without bound IgG antibodies, was improved by "electron spectroscopic imaging", a recently developed mode of electron microscopy.

A detailed electron microscopic study of solubilized oxaloacetate decarboxylase [13] provided insights into the architecture of the α subunit, which is known to carry the (single) catalytic site of the enzyme. The localization of the active site on the surface of the α subunit was performed by affinity labelling of the biotin moiety (which is the CO₂ carrier oscillating between two subsites) with avidin. It could be shown unequivocally that the biotin is located in a cleft, separating two domains of the α subunit, close to the site of contact of the α subunit with the membrane-integrated $\beta\gamma$ complex. Although oxaloacetate decarboxylase is a

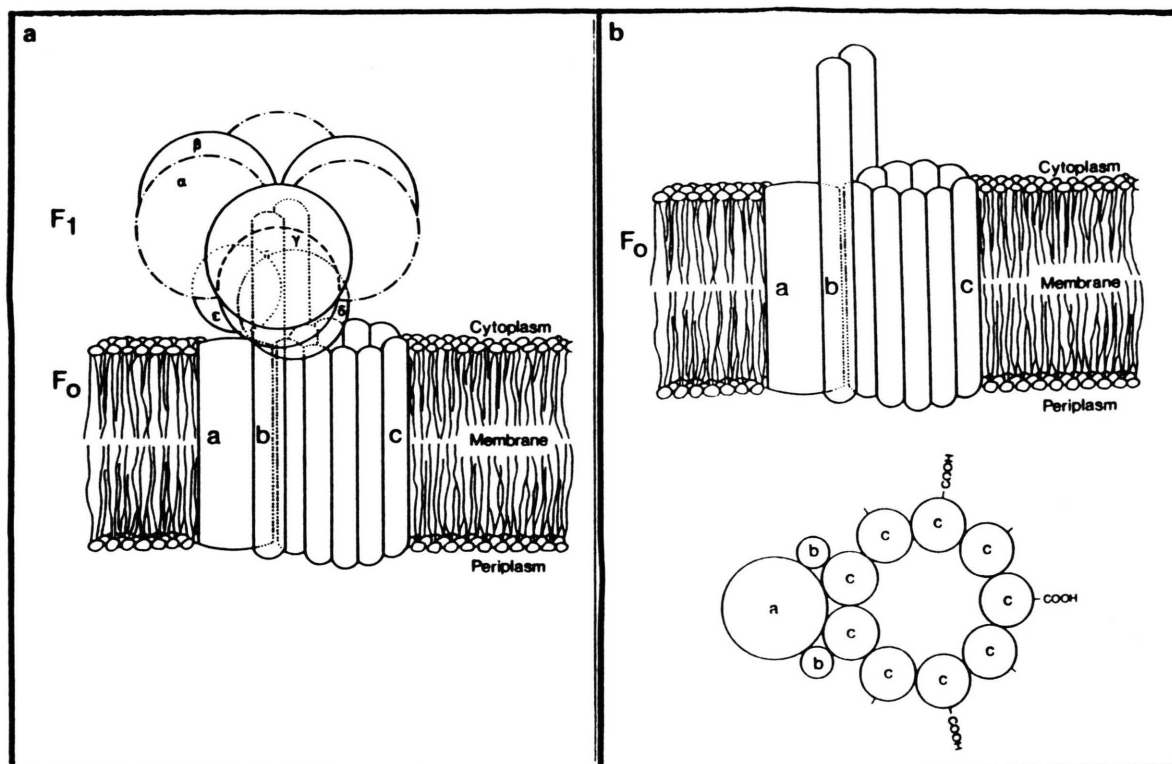


Fig. 2. Diagrammatic views of the F_0F_1 -ATPase (a) and the F_0 part of this enzyme (b) in *Escherichia coli*. Top: side-on view; bottom of b: face-on view. α , β , γ , δ , ϵ , subunits of F_1 ; a, b, c, subunits of F_0 . (From ref. [12].)

membrane protein, it appears to have a remarkable degree of similarity at the level of ultrastructure with soluble biotin-containing enzymes, such as pyruvate carboxylase [14]. Features common between oxaloacetate decarboxylase and soluble biotin enzymes include the location of the active site in a cleft (*i.e.* between two domains of a subunit [15, 16]). There are at least two evolutionary scenarios which could lead to this similarity; one of these, which has been previously discussed [14], is that this common architecture is a result of gene fusion events which have occurred during the evolution of biotin enzymes. The other rationalization is that the biotin-containing cleft has been conserved between membrane-bound and soluble biotin enzymes, and that oxaloacetate decarboxylase illustrates an additional degree of sophistication over the soluble biotin enzymes in that in this case the catalytic reaction has been coupled to cellular energy conservation (Fig. 3).

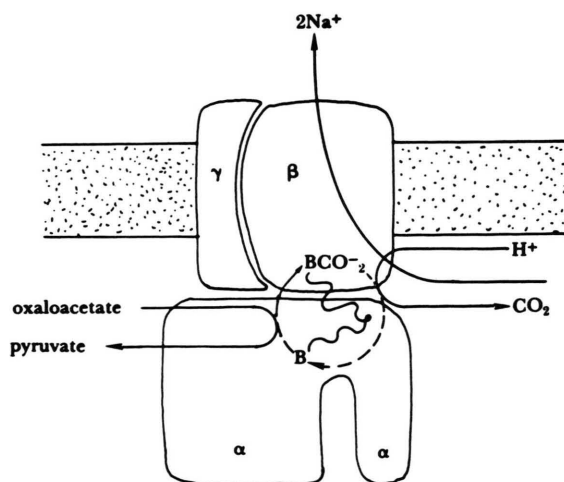


Fig. 3. Hypothetical model linking structure and function of the oxaloacetate decarboxylase subunits (α , β , γ). For further explanation see text. (From ref. [8].)

*Growth on organic substrates:
intracellular hydrogen cycling for the generation
of a proton gradient*

The ability of bacteria of the genus *Desulfovibrio* to utilize either organic or inorganic electron donors (such as H_2 or CO) for the reduction of sulfate to sulfide is well known [17]. For growth on organic substrates, Odom and Peck [18] postulated a mechanism of energy conservation involving intracellular hydrogen cycling. Hydrogen is assumed to be produced in the cytoplasm from organic substrates, to then diffuse across the cytoplasmic membrane, and to be oxidized by hydrogenases in the periplasm to protons and electrons. These electrons are transferred into the cytoplasm and consumed in the reduction of sulfate, a process known as vectorial electron transfer [19]. The net effect is the transfer of eight protons from the inside to the outside of the cytoplasmic membrane for each molecule of sulfate reduced to sulfide.

A requirement for hydrogen cycling is the presence of hydrogenases in both the periplasm and the cytoplasm [20]. It was suggested that a (NiFeSe) hydrogenase is located at the inside of the cytoplasmic membrane and that this enzyme is responsible for hydrogen production in the cytoplasm. The periplasmic counterpart was postulated to be a (NiFe) hydrogenase, located at the outside of the cytoplasmic membrane. This latter enzyme was assumed to be responsible for the activation of hydrogen for the reduction process described above. A third hydrogenase, a well characterized soluble (Fe) hydrogenase, is known to be present in the periplasmic space (Fig. 4). In order to substantiate the concept of hydrogen cycling, immuno electron microscopy was performed on cells of *Desulfovibrio vulgaris* [21]. Polyclonal antibodies against each of the nickel-containing enzymes were used in both pre- and post-embedding labelling techniques. Ferritin was applied to detect one of the labels, via Fe-specific electron spectroscopic imaging.

When post-embedding labelling on ultrathin sections was performed, the (NiFe) hydrogenase was located along the cell periphery. However, this technique is not suitable for the investigation of membrane "sidedness" of an enzyme, necessitating the application of pre-embedding immunolabelling techniques. Spheroplasts were labelled, prior

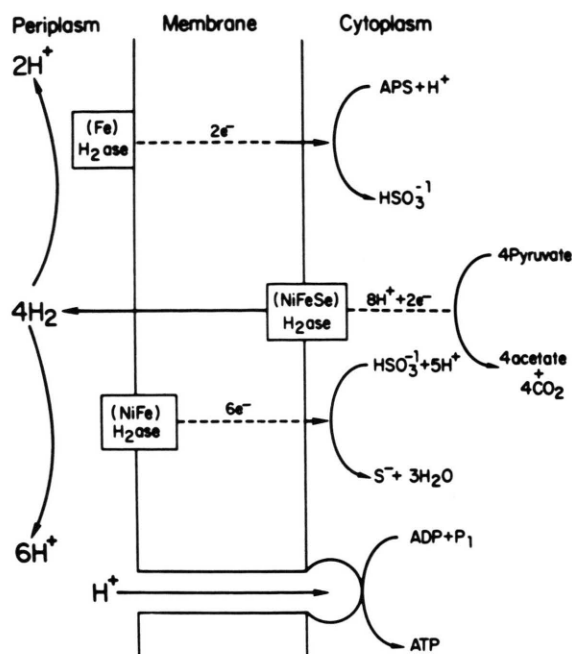


Fig. 4. Diagrammatic representation of hydrogen cycling and the cellular location of the (NiFe) and (NiFeSe) hydrogenases in *Desulfovibrio vulgaris*. APS, adenosine 5'-phosphosulfate. For further explanations see text. (From ref. [21].)

to embedding, with anti-(NiFe)-hydrogenase antibodies raised in rabbits; these antibodies were localized using secondary (anti-rabbit IgG) antibodies conjugated to ferritin as electron-dense marker. After application of this technique, ferritin molecules could be detected at the outer aspect of the cytoplasmic membrane, in areas where the outer membrane was detached from the cytoplasmic membrane and murein as a result of lysozyme treatment. Washing of spheroplasts prior to application of antibody led to a loss of labelling, indicating a weak interaction between the (NiFe) hydrogenase and the membrane. By post-embedding labelling, the (NiFeSe) hydrogenase was also found along the cell periphery. By pre-embedding labelling, however, anti-(NiFeSe)-hydrogenase antibodies did not label the periplasmic side of the cytoplasmic membrane. This demonstrates that the (NiFeSe) hydrogenase is located at the cytoplasmic side of the cytoplasmic membrane, and there are no indications for a transmembranous location of this enzyme. These immuno electron microscopic studies unequivocally confirmed the

spatial separation of the (NiFe) and (NiFeSe) hydrogenases by the cytoplasmic membrane of *D. vulgaris*, which is a requirement of the hydrogen cycling model and the bioenergetic involvement of molecular hydrogen in the generation of a proton gradient.

Conclusion and Perspectives

Though structural organization of the living cell is a momentary state, reflecting a momentary functional situation, principles governing cellular structure-function interaction are evident. Bacterial enzyme systems involved in cell energetics are examples which demonstrate that not only in cells of higher organisms, but also in prokaryotes, organization – though less complex – is the prerequisite for cellular activities.

It is hoped that physiological, biochemical and enzymological approaches used in the analysis of bacteria are further complemented by application of recently established ultrastructural investigation techniques [22]. The goals of the development of these methods have been: reduced artefact formation, visualization of cells in “quasi-living” state, and high specificity with respect to the identification and localization of cell components. All together, they are suited to add a new quality to the attempts usually made when living cells, their constituents and the dynamics inherent in their functional states are to be analyzed.

Acknowledgements

Work in the authors' laboratory was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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