But if cultivation is extended further, what may happen to Sangai in future?

The author recommends extending such cultivation for better economy. But that is not without problems. The solution to India's food and economic problems is not increasing cultivation and technology, but efforts on a war-footing for immediate control and gradual reduction of human population and equitable distribution of resources. Enough of war on nature. We should restrict our own numbers and lifestyle so as to live sustainably by milking only milk and not blood too from the cow called nature.

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NEWS

SIRO numbers swell: 519 and counting

The number of non-commercial Scientific & Industrial Research Organizations (SIROs) in the country has swelled to 519. The Department of Scientific and Industrial Research (DSIR)'s September 1997 Directory of Recognized SIROs lists 37 SIROs in agricultural sciences, 159 in medical sciences, 188 in natural and applied sciences, 113 in social sciences, and 22 universities/colleges. Covered in this latest Directory are: (a) Associations, i.e. societies registered under the Societies Registration Act, 1860 or any such Act passed by the State Government, with the objective of conducting scientific and/or industrial research; (b) R&D Companies incorporated under section 25 of the Companies Act, 1956 and setup for engaging in R&D activities; (c) Institutions with adequate facilities to conduct scientific and/or industrial research and whose main objective is the conduct of scientific and/or industrial research; (d) Professional bodies whose objective is the conduct of scientific and/or industrial research; (e) Universities established or incorporated by/or under a Central or State Act and including institutions declared under section 3 of the University Grants Commission Act, (1956) and (f) Colleges, affiliated to universities, that carry-out scientific research in specific disciplines.

DSIR is the nodal government department for granting recognition to SIROs. Such recognition entitles SIROs to tax benefits under Section 35(1)(ii)(iii) of the Income Tax Act, 1961 and also Customs Duty exemption and waiver of Excise Duty.

RESEARCH NEWS

The different locked states of an allosteric membrane channel protein unlocked

S. K. Sikdar

Allosteric proteins ('allo' – meaning other) are proteins that have specific sites to which physiologically important molecules or ligands bind to regulate the activity of the protein, and these sites are different from the sites involved in catalytic activity. A ligand binds to a particular conformation of the protein and not to others. When a protein is a complex of subunits, and the subunits occur in clusters, with each subunit having a ligand-binding site, cooperative changes occur within the protein molecule in a complex fashion, such that binding of the first ligand makes the binding of the second ligand easier. One such protein is the oxygen-carrying haemoglobin and is used in text books of biophysical chemistry to explain how interaction of the protein with many ligands modulates its activity. An important theoretical model to explain how an allosteric protein like haemoglobin is regulated, is the MONOD-WYMAN-CHANGEUX (MWC) model[1]. In this model, a protein is considered to consist of n identical and independent protomers (in the original terminology), a protomer being a structural unit (subunit) which has a binding site for the ligand. A protomer can exist in two reversibly equilibrating conformational states, a ligand bound and an unbound state. The activity of the protein occurs due to concerted conformational changes in the protomers, and helps to explain the hyperbolic saturation curve for binding of a ligand to identical and independent binding sites on the protein. The MWC model does have limitations, however, and cannot fully explain all the experimental

data. An alternative model to explain the function of allosteric proteins is the sequential model of Koshland and his colleagues[2], which suggests that binding of the ligand produces change in the structure of the protein sequentially, and transition from a non-functional to a fully functional protein occurs through a series of intermediate conformational states. If we consider a protein with 4 subunits, binding of the ligand would cause a reversible conformational change in one subunit, the second ligand would next cause a conformational change in the second subunit, and when all the subunits are ligand bound, the protein is fully functional. The model accommodates a wide variety of binding behaviour. A more general scheme has also been proposed[3], and for a 4-subunit protein, the scheme predicts 25 intermediate forms of the protein. The intermediate conformational forms predicted by the MWC model and the sequential model of Koshland are accommodated in this scheme.

Experimental identification of the intermediate forms where the protein is in different structural and thereby functional states has eluded both structural biologists and biophysical chemists. The problem is simply related to the way the protein is treated – an ensemble of molecules such as in a test-tube where individual protein molecules can exist in different conformational forms, and it is difficult to capture the protein at different time frames in the scale at which ligand binding and the conformational change in the individual subunit occurs.

Allosteric proteins also occur in cell membranes and have been occupying a lot of interest, but the people who look at these proteins are electrophysiologists – people interested in interpreting protein function by monitoring currents that are carried by the ions that are transported through ion channel proteins sitting in membranes. By monitoring the current flow through a single protein molecule in the membrane, the recent work of Ruiz and Karpen[4] reported in *Nature*, seems to indicate that they have indeed been able to successfully capture the intermediate functional forms of the protein which has eluded people working with a molecule like haemoglobin for decades. Their work is on the CNG (cyclic nucleotide gated) ion channels expressed in xenopus oocyte. This channel protein transduces sensory information such as light, odour and taste into electrical signals. In order to convert sensory input to an electrical signal, the channel protein must open. An allosteric mechanism has to be inherently built in the protein if it were to discriminate the different gradations in sensory information. Here, the function of the protein is similar to haemoglobin which has to detect different partial pressures of oxygen, such that the molecule functions differently in the planes than at an altitude.

The CNG channel protein is a tetramer[5] consisting of 4 identical and independent subunits, and if we consider ligand binding to each of these, using the models described, it should be possible to see the intermediate forms of the protein in different functional states. If the protein contains 4 identical subunits, with identical binding sites, there can be different possibilities, since each subunit can be in two states, 'closed' or 'open', the transition from the closed to the open state being brought about by cGMP. The channel opens when all the 4 subunits are in the open state. The transitions from the closed 'non-conducting' state of the channel protein with 4 subunits to the fully conducting state is illustrated schematically in Figure 1*A*. When the level of cGMP increases to saturating levels, the channel opens in a 'cooperative fashion', and the suggestion for this has come from a sigmoid-shaped activation curve.

To see the intermediate forms of the protein in different functional states, it is important to monitor the activity of a single protein molecule. This is achievable using the inside-out configuration of the patch-clamp recording technique, a technique, which has brought a revolution in the way single living cells and their functioning are probed[6], and the resolution allows one to observe the intermediate functional steps which manifest as sub-conductance states leading to complete channel opening. The second important requirement is that one should be able to create partially ligand bound channels. This they have achieved by using the photoaffinity analogue of cGMP, viz. 8-p-azidophenacylthio-cGMP (APT-cGMP). The cGMP can be covalently and thereby permanently tethered to the intracellular face of the channel protein facing the bath solution containing APT-cGMP, by exposure to UV light[7]. With the ligand attached covalently, maximal activation of the channel should require lower concentrations of free cGMP, and this should be reflected in the dose-response curve. The slope of the dose-response curve should become shallower (lower Hill coefficient), since the unoccupied binding sites are reduced, compared to control with no covalently bound cGMP. This is shown schematically in Figure 1*B*. With APT-cGMP in the bath solution, the number of UV exposures was increased from a single to multiple exposures as shown schematically in Figure 1*A*, and a dose-response curve in the presence of free cGMP in the bath solution done. The dose-response done in the presence of free cGMP following a fixed number of exposures, 1, 2, 3, or 4 clustered into 4 distinct and discrete groups consisting of control and 3 different shifts in the activation to lower concentrations of cGMP with increase in the number of UV exposures. The dose-response curve was fit to a Hill equation, and shifts in the Hill coefficient (from 2.9 for control which shifted to 1.9, 1.4 and 1.1 with increase in the number of UV exposures) were taken as evidence for covalent attachment of cGMP as indicated in Figure 1*A*. The Hill equation was initially derived for an ideal case of completely cooperative binding at n sites. Completely cooperative binding implies that the 4th binding site for a 4 subunit CNG channel, would have no affinity for cGMP until all the other 3 binding sites are filled. Hill plots are used by biochemists to plot experimental data for systems in which the cooperativity is incomplete. Thus, the experimentally determined slope of the Hill plot (n_{Hill}) is usually less than n, the number of binding sites. The non-integral values of the Hill coefficient reported by Ruiz and Karpen[4] are therefore not very surprising.

It is tempting to imagine that a single cGMP occupied channel might represent a conformation similar to that in Figure 1*A*(*b*), while a channel with 3 cGMP bound may represent a conformation shown in Figure 1*A*(*d*), and this would result in sub-conductance states shown schematically in Figure 1*C*, $n = 1$ and $n = 3$ respectively. At saturating

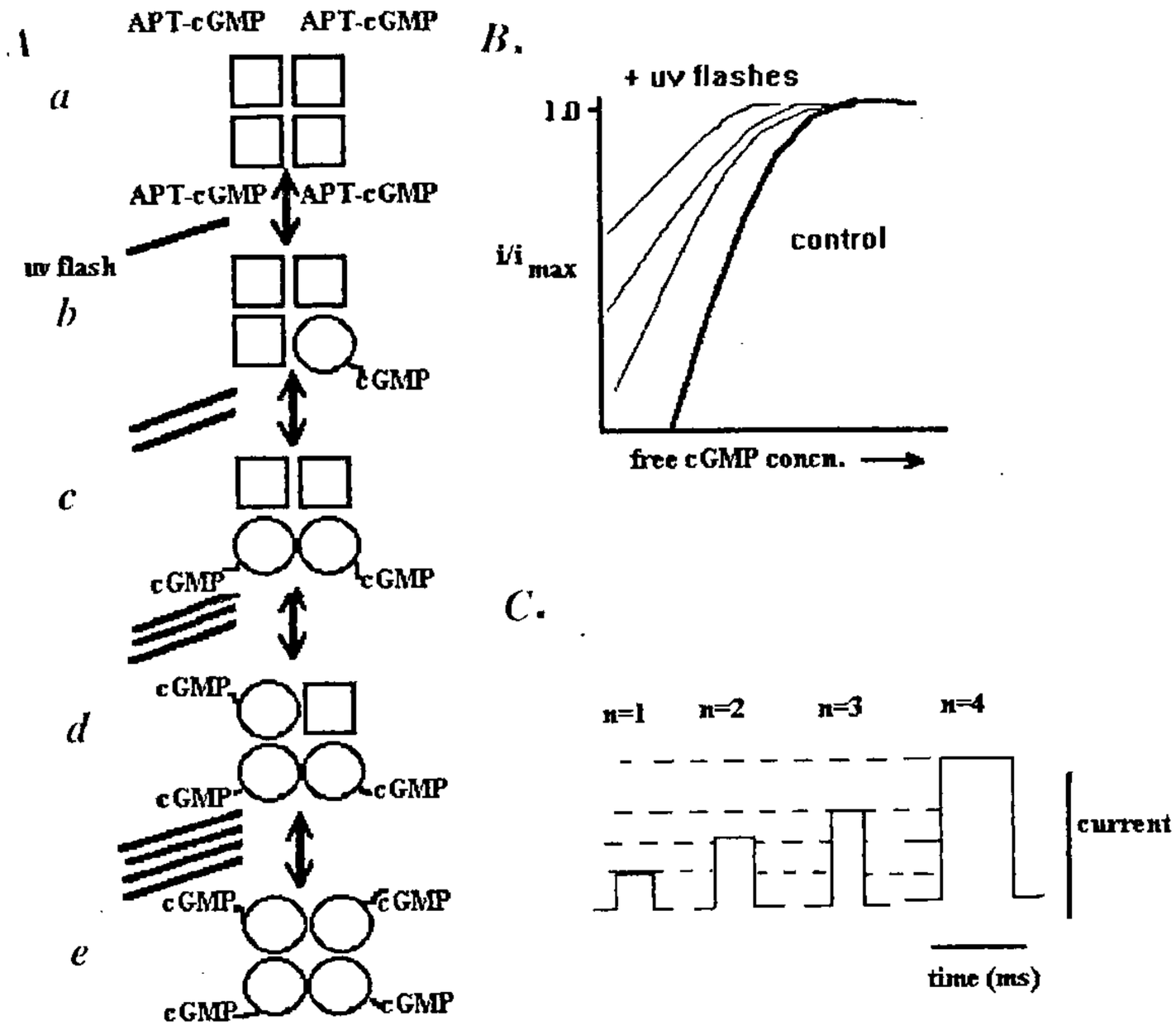

Figure 1. *A*, An allosteric scheme for covalent binding of cGMP to CNG (cyclic nucleotide gated) ion channel. A single CNG protein molecule has 4 subunits in unliganded state (squares) which switch from a 'closed' or non-conducting, to an 'open' or conducting state (circles), when cGMP is covalently bound following UV exposure of APT-cGMP (see text). The number of cGMP molecules covalently bound to the protein can be increased by increasing the number of UV-exposures (*a* to *d*). *B*, Predicted shift in the dose-response curve and thereby the Hill-coefficient following UV exposure of APT-cGMP in the presence of free cGMP in the bath solution facing the intracellular side of the channel. If a channel is in the conformation shown schematically in 1*A*(*d*) for instance, with 3cGMP covalently bound to 3 subunits, then only 1 free cGMP molecule can bind to the channel protein, thereby causing a discrete leftward shift in the dose-response curve. i/i_{max} is a measure of spontaneous activity (mean current/saturating current at saturating concentration of cGMP). *C*, The different electrophysiologically observed conductance states with different numbers of cGMP molecules (n = 1, 2, 3, 4) bound to a single channel protein. A conformational state shown in 1*A*(*c*) with 2cGMP molecules covalently bound, for instance would result in the subconductance state (n = 2), while a channel with all subunits covalently linked to cGMP would result in the highest conductance state (n = 4). The subconductance states occur because the transport of ions through the pore is restricted when all the subunits have not undergone the conformational switch shown in 1*A*.

concentrations of cGMP, the channel mainly showed the full conductance state, while at sub-saturating concentrations of cGMP when the channel is not fully open, sub-conductance states were observed in single CNG channel recordings. How realistic are the imaginary intermediate conformational forms, and can a change in a single subunit from 'closed' to 'open' conformation really cause a decrease in ionic permeability which is manifested as sub-conductance states shown schematically in Figure 1*C*, by restricting the pore size? Careful experiments performed by Goulding *et al.*[8] have shown that CNG channels which transduce visual and olfactory impulses have pore diameters as large as 5.9 Å and 6.4 Å respectively, while the crystal radii of cations which are transported through the channel pore are much smaller, the crystal radii of Na^+, K^+ and Ca^{++} ions being 0.98, 1.33 and 0.99 respectively.

While substates or subconductance states at low subsaturating concentrations of free cGMP have been proposed to be due to partial liganded channels by Taylor and Baylor[9], the evidence for this has indeed come from the elegant experiments of Ruiz and Karpen[4].

The functional signature of the channel protein in the different covalently linked ligand bound states was obtained by analysing how the channel opens with the cGMP covalently tethered to the channel protein with either a single cGMP bound or with 2, 3 or 4 cGMP molecules bound to the corresponding number of subunits. The studies were done with free cGMP absent in the bath solution but with UV exposure of APT-cGMP. Probability density histograms of the amplitude of current events which is a reflection of the time spent in each current level were made. While a single ligand bound channel opened with a very low probability (9.6×10^{-6}), channel with 2 and 3 cGMP molecules bound showed open probability of 0.0097 and 0.33 respectively, while a channel with fully occupied cGMP showed open probability of 1. A fully ligand bound channel showed openings favouring the maximal conductance state, while triply liganded channels showed highest probability of opening to the subconductance states. A singly or doubly bound CNG channel showed negligible subconductance states. This provides information about how the channel might actually function as a switch. If the channel showed subconductance states with 1 or 2 cGMP molecules, it would indicate that the channel functions as a slow switch. The situation is akin to lighting an electric bulb by switching the voltage from 0 to 220 volts using a fast switch almost instantaneously and increasing the voltage using a rheostat. The CNG channel functions like a fast switch, such that when 3 cGMP molecules are bound, the channel opens partially while when 4 cGMP molecules are bound, the channel opens completely, the activation of the channel being controlled by a threshold concentration of cGMP. How does the sensory cell then sense gradations in sensory stimulus such as light and smell, a dim light versus an intense bright light for example? This is probably brought about by controlling how many CNG channels are switched on, since each sensory cell would have a fixed number of CNG channels in its plasma membrane, and the number of cGMP molecules inside the cell is what is affected by the intensty of the sensory stimulus.

Electrophysiologists have, in general been a poorly understood lot, and their contribution to biological understanding apart from how electrical impulses propagate in living systems, have not been taken with seriousness. It is indeed very heartening to note that the very probing and kissing of cells with glass capillaries with tiny tips filled with ionic salt solutions, connected to complicated electronic gadgetry, is providing important clues to classical biochemists and biophysical chemists how a protein like haemoglobin might actually function at the single molecular level as has been demonstrated by the elegant experiments of Ruiz and Karpen[4]. Further, the experiments also go to show that while theoretical modelling of protein behaviour of the kind suggested by the MWC cooperative model or the sequential model of Koshland provide insights and the required lead, a well conceived and carefully-performed experiment does provide the evidence and helps identify the right model that actually operates in a particular biological system. The evidence provided by Ruiz and Karpen[4] seems to indicate that the intermediates in the activation pathway of the CNG channels are more akin to the 'Koshland intermediates'[2].

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ACKNOWLEDGEMENTS. I thank Ambarish S. Ghatpande for providing comments.

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SCIENTIFIC CORRESPONDENCE

Nitrogenase activity in novel vesicles of *Frankia*

For inoculation of actinorhizal plants, frankial fragments are added around the roots of these seedlings. About 40% of *Frankia* added as inoculum cause root hair deformations in *Alnus nepalensis* Don. and this is generally followed by the formation of root nodules. It is not understood what happens to *Frankia* that is in the vicinity of root hairs but is unable to infect them. This work with one endophyte demonstrates that these rhizospheric *Frankia* form vanadium nitrogenase in novel structures and opt for a non-symbiotic mode of survival.

Seeds of *A. nepalensis* were surface sterilized, germinated and transferred to sterile vials. These vials contained a sterile filter paper placed on a sterilized glass microslide. The filter paper was moistened with Hoagland[1] nutrient solution and seedlings placed over the filter paper. Seedlings were then maintained at 21–24°C and with a photoperiod of 16/8 hours day/night. Each vial was given one ml of N-free Hoagland solution once a week. Two-week-old seedlings were inoculated with a small volume (300 µl) of homogenized AnpCh57. This strain had been isolated from root nodules of *A. nepalensis* growing around Cherrapunji (Meghalaya, India) and cultures grown in defined propionate medium[2] (DPM) for two weeks before inoculation.

Ten days after inoculation, seedlings along with filter paper were taken out of vials and observed. On the growing root hairs and in close vicinity to them were hyphae of AnpCh57 bearing roundish structures (Figure 1 *a*). These structures continued to enlarge and on day 15 reached a maximum diameter of 2.8 ± 0.4 µm. At this stage the hyphae bearing these structures also started to lyse (Figure 1 *b*). It is possible that since the vials contain only Hoagland nutrient solution and root exudates, it is unable to support growth of AnpCh57. AnpCh57 requires a complex medium like Qmod[3] for its optimal growth. It should be added here that hyphae and ball-shaped structures were not contaminants as assessed by routine techniques for identifying *Frankia*.

When these ball-shaped structures along with hyphae were transferred to Qmod or DPM, before onset of degeneration, hyphae started producing normal spherical vesicles. Ball-shaped structures were formed only in DPM lacking molybdenum and containing vanadium. However, the ultrastructure of these ball-shaped structures was similar to that of spherical vesicles.

Nitrogenase activity was measured with AnpCh57 bearing ball-shaped structures immediately after subculture in DPM and Qmod. These cultures were not effective in fixing atmospheric nitrogen. However, when molybdenum was deleted from DPM and supplemented with vanadium, nitrogenase activity was observed (Table 1). Thus this nitrogenase is not active in the presence of molybdenum or the genes of nitrogenase complex are regulated by an unknown mechanism. As seen from the table, vanadium nitrogenase is less efficient than molybdenum nitrogenase at 28°C. Similar results have been obtained with vanadium nitrogenase of other nitrogen-fixing bacteria and explained to be due to only 50% of electron flux going to the formation of NH_3 (ref. 4).

Cultures of AnpCh57 were then transferred to sterile sand/soil. Obse-