pH REGULATION OF LUCIFERASE ACTIVITY IN DINOFLAGELLATES INVOLVES A NOVEL ENZYMATIC MECHANISM

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INTRODUCTION

Regulation of luciferase (LCF) activity in dinoflagellates is unique, without precedent in other enzymes. In brief, it involves control of substrate binding. At pH 8 its conformation prevents substrate from binding. At pH 6 the LCF conformation changes, allowing the tetrapyrrole luciferin (LH₂) to access the binding site. The reaction then occurs giving a bright, brief flash of less than 100 msec. In at least one species, (*Lingulodinium polyedrum*, formerly *Gonyaulax polyedra*), regulation is enhanced by the action of a second protein, luciferin binding protein (LBP), which binds luciferin at pH 8 but releases it at pH 6.¹ Such a large and rapid pH jump is possible by virtue of the fact that the light emitting system is contained in small (~0.5 μ m), cortically located novel organelles named scintillons. They occur as outpocketings of the cytoplasm, projecting into the cell vacuole, connected like a balloon (Fig. 1), and containing only LCF, LH₂ and LBP. They can be identified by

Figure 1. Schematic scintillon

immunolabeling with antibodies raised against LCF or LBP, and visualized by their bioluminescent flashing following stimulation, which is co-localized with the fluorescence of luciferin. Based on the effects of pH on the activities of purified LCF and LBP, and also on isolated scintillons, *in vivo* flashing was postulated to result from a transient pH change in the scintillons, triggered by a mechanically initiated action potential in the vacuolar membrane which opens ion channels that allow protons from the acidic vacuole to enter. As they are effectively isolated from the parent cytoplasm, very few protons are needed to change the pH from 8 to 6.

THREE ACTIVE SITES IN A SINGLE PROTEIN

The full-length *lcf* cDNA (4,037 bp) has an open reading frame of 3,723 bp and encodes the 136,994 Da protein (Fig. 2), being comprised of three contiguous intramolecularly homologous domains, D1, D2 and D3, with no intervening nucleotides.² Upon cloning and expressing each domain individually, it was found that each is catalytically active as a luciferase. The domains are ~ 75% identical overall, and ~95% identical in the more central active site regions.

Figure 2. The structure of *L. polyedrum* luciferase

We hypothesize that the three contiguous luciferase domains, with their catalytic sites associated separately with an LBP and LH₂, are structured as a supra molecular unit, and that scintillons contain many such units. This structure is itself unique. The presence of three repeated conserved sequences in one enzyme molecule is not unprecedented, but to our knowledge, this is the only enzyme in which each of the domains has been shown to be separately active, and to be serviced by a protein holding the substrate. A possible reason for such a structure is that the presence of three active sites on a single molecule allows activity to be greater without an increase in the osmotic pressure of the scintillon.²

It was early observed that ~35kDa proteolytic fragments of the soluble luciferase have activity, which can now be interpreted as coming from individual domains. But fragments lacked pH control; might this be a property of the full-length molecule? No. Peptides embracing a single full domain exhibit pH activity curves similar to that of the full-length molecule. However, single domain peptides, in which about 50 to 70 N-terminal amino acids are absent, exhibit higher activity at pH 8, like the proteolytic fragments.

CONSERVED HISTIDINES

Inspection of the N-termini of the three domains revealed four histidines conserved in all three domains, and it was considered that these might be responsible for the low activity at pH 8. Indeed, their replacement singly by alanine, using site-directed mutagenesis, resulted in luciferases with much higher activity at pH 8, and even greater with multiple replacements (Table 1).³ From this result we proposed that pH-dependent charge changes of these non-catalytic histidine residues regulate luciferase activity through conformational shifts in protein structure.

The four conserved histidines are found in all seven species examined

We cloned and sequenced the *lcf* genes from six additional species of luminous dinoflagellates and found that the histidine residues identified in *L. polyedrum* are conserved in all three domains of all seven species (Fig. 3), indicating that the mechanism of activity regulation by pH is similar in all.

Table 1. Activities of mutants

Mutants	Activity at pH 8*
WT	4
H35A	43
H45A	30
H60A	8
H66A	9
H1,2	82
H1,2,3	108
H1,2,3,4	98

* % of activity at pH 6.3

	30	40	50	60
Aa_D1	FKDGLHKPKW	DSEGLHKPHT	IGGKTYETGF	HYLLEAHELG
At_D1	FKDGLHKPKW	DSEGLHKPHT	IGGKTYETGF	HYLLEAHELG
Lp_D1	FKGGLHRPKF	DSEGLHKPHT	SGGKTYETGF	HYLLEAHELG
Pf_D1	FKGGLHKPNF	HSEGLHMPHT	SGDKTYDTGF	HYLLEMHDLG
Pl_D1	FRGGLHQPKF	HTDGLHMPHT	SGEKTYYTGF	HYLLEVHDLG
Pn_D1	FKGGLHKPNF	HSEGLHMPHT	SGGKEYETGF	HYLLEMHDLG
Pr_D1	FKGGLHKPDW	DKEGLHKPHT	IGGKTYDTGF	HYLLEAHDLG
Aa_D2	FKDGLHQPKF	HEEGLHKPME	AGGRVYTTGF	HYLLEAHELG
At_D2	FKDGLHQPKF	HEEGLHKPME	AGGRVYTTGF	HYLLEAHELG
Lp_D2	FKNGLHAPNF	HDDGLHKPME	AGGRVYSTGF	HYLLEAHDLG
Pl_D2	FEDGLHKPKF	HDDGLHKPME	AGGRVYETGF	HYLLEAHELG
Pn_D2	FEGGLHKPKF	HDDGVHGPMT	AGGKEYETGS	HYLLEAHELG
Pr_D2	FEGGLHKPKF	HEEGLHKPME	AGGRVYTTGF	HYLLEAHELG
Aa_D3	FKNGLHQPTF	HAEGHLKEME	VNGKTYGSGF	HYLLECHELG
At_D3	FKNGLHQPTF	HPGPHKEME	VNGKKYDSAF	HYLLECHELG
Lp_D3	FKNGMHKPEF	HEDGLHKPME	VGGKKFESGF	HYLLECHELG
Pf_D3	FKDGLHQPTF	HEEGLHKPVE	AGGRVYETGF	HYLLECHELG
Pl_D3	FKDGMHQPTF	HDEGLHKPME	AGGRVFESGF	HYLLECHELG
Pn_D3	FQDGLHQPTF	HEEGLHKPVE	AGGRVYETGF	HYLLECHELG
Pr_D3	FKNGLHQPTF	HPGAKHEME	VNGKTYESGF	HYLLECHELG

Figure 3. Alignments in the region of the conserved histidines of the three domains of the seven luciferases. Pl, *Pyrocystis lunula*; Pn, *P. noctiluca*; Pf, *P. fusiformis*; At, *Alexandrium tamarense*; Aa, *A. affine*; Pr, *Protoceratium reticulatum*.

Structure of domain 3 of *L. polyedrum* giving the location of histidines

We determined the 3-D structure of LCF domain 3 of *L. polyedrum*.⁴ The histidines are in a region where protonation could regulate substrate binding (Fig. 4).

Figure 4. Crystal structure, domain 3

The structure at pH 8 reveals a putative active site pocket, but in that structure there is neither enough room to bind the substrate nor an opening to the interior of the barrel. The presence in the pocket of several polar residues that could participate in catalysis, including histidine, tyrosine, and glutamate, is suggestive of an active site. Site directed mutagenesis of five such residues has been found to result in loss of luciferase activity. Proline rich loops surround the β -barrel and may serve to stabilize the active site by tying the protein together like a standing rib roast.

MOLECULAR MECHANISM OF pH REGULATION

Our hypothesis regarding the mechanism for the pH-controlled regulation of LCF activity is that the histidine residues make contacts that stabilize the N-terminal domain and the helix-loop-helix. Disruption of such contacts by protonation in the wild-type, or in his to ala mutants at pH 8, causes the N-terminal domain and the helix-loop-helix to move and open the catalytic active site. This appears to be altogether novel in enzyme chemistry as a mechanism for regulation of activity.

The D3 domain structure reveals that these histidine residues are at an interface with the helix-loop-helix motif that covers the putative active site (Fig. 4). H899 acts as a core residue in the N-terminal subdomain forming a hydrogen bond with Y925. There are very few interactions that tether the N-terminal subdomain to the rest of the protein and the hydrogen bond of H899 to the main chain carbonyl of V1087. This is an important residue, as it is positioned on a stable turn of the β -barrel and may serve to anchor the N-terminal subdomain to the β -barrel. H909 is in van der Waals contact with A1052 and forms a hydrogen bond with the main chain carbonyl of L1050, both of which lie in the loop of the helix-loop-helix. H924 forms a hydrogen bond with S921 and is also in van der Waals contact with I1045 of the helix-loop-helix. Lastly, H930 forms a hydrogen bond with Q1037 of the helix-loop-helix and is resting in a hydrophobic pocket created by A1088, A1038 and M1070.

Preliminary molecular dynamics calculations (Sybyl, Tripos engine) indicate that the N-terminal domain and the helix-loop-helix are more mobile than the rest of the protein under the conditions of the simulation. In addition, the residues in this area of the protein have higher overall B-factors ($39 \text{ \AA}^2$) when compared with the rest of the protein ($23 \text{ \AA}^2$). A model of the H899A, H909A, H924A and H930A variant of D3 was created using the X-ray coordinates of the native D3 structure and used in a 1 ns molecular dynamics simulation at 300K. During the simulation, the N-terminal domain and helix-loop-helix moved away from each other about $5 \text{ \AA}$ and both away from the protein about $10 \text{ \AA}$. The rest of the protein remained stable and deviated less than $1 \text{ \AA}$ from the starting model. These movements served to open up a solvent-accessible channel to the putative active site. Three separate Gly-Gly sequences within the N-terminal domain and the helix-loop-helix served as hinges about which the chains rotated (Fig. 4).

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