Journal of Biochemistry and Molecular Biology, Vol. 39, No. 5, September 2006, pp. 578-585

JOURNAL OF Biochemistry and Molecular Biology

cDNA Cloning, Expression and Homology Modeling of a Luciferase from the Firefly *Lampyroidea maculata*

Abdo Rahman Emamzadeh[1], Saman Hosseinkhani[1,*], Majid Sadeghizadeh[2], Maryam Nikkhah[3], Mohammad Javad Chaichi[4] and Mojtaba Mortazavi[1]

[1]Department of Biochemistry, Faculty of Basic Sciences, Tarbiat Modarres University, Tehran, Iran
[2]Department of Genetics, Faculty of Basic Sciences, Tarbiat Modarres University, Tehran, Iran
[3]Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran
[4]Department of Chemistry, Mazandaran University, Babolsar, Iran

Received 7 April 2006, Accepted 23 May 2006

The cDNA of a firefly luciferase from lantern mRNA of *Lampyroidea maculata* has been cloned, sequenced and functionally expressed. The cDNA has an open reading frame of 1647 bp and codes for a 548-residue-long polypeptide. Noteworthy, sequence comparison as well as homology modeling showed the highest degree of similarity with *H. unmunsana* and *L. mingrelica* luciferases, suggesting a close phylogenetic relationship despite the geographical distance separation. The deduced amino acid sequence of the luciferase gene of firefly *L. maculata* showed 93% identity to *H. unmunsana*. Superposition of the three-dimensional model of *L. maculata* luciferase (generated by homology modeling) and three dimensional structure of *Photinus pyralis* luciferase revealed that the spatial arrangements of Luciferin and ATP-binding residues are very similar. Putative signature of AMP-binding domain among the various firefly species and *Lampyroidea maculata* was compared and a striking similarity was found. Different motifs and sites have been identified in *Lampyroidea maculata* by sequence analysis. Expression and purification of luciferase from *Lampyroidea maculata* was carried out using Ni-NTA Sepharose. Bioluminescence emission spectrum was similar to *Photinus pyralis* luciferase.

Keywords: AMP-Binding domain, Firefly, Homology modeling, *Lampyroidea maculata*, Luciferase, Sequence analysis

*To whom correspondence should be addressed.
Tel: 98-21-8801-1001; Fax: 98-21-8800-9730
E-mail: saman_h@modares.ac.ir

Introduction

Firefly luciferase (EC 1.13.12.7) is a well-characterized enzyme that is responsible for the bioluminescence reaction. It catalyzes the oxidation of firefly luciferin with molecular oxygen in the presence of ATP and Mg^{2+} to emit yellow-green light (McElroy, 1969; White *et al*., 1971; DeLuca, 1976; Wood, 1995). The initial reaction catalyzed by firefly luciferase is the formation of luciferyl adenylate with the release of inorganic pyrophosphate. The luciferase-bound luciferyl adenylate reacts rapidly with molecular oxygen to give light, CO_2, AMP and oxyluciferin.

Deluca and colleagues were pioneers in cloning and sequencing of the first species of luciferase, *P. pyralis* (De Wet *et al*., 1985). Since then, luciferase genes from various firefly species have been isolated (Alipour *et al*., 2004; Viviani *et al*., 2004 and references therein) and the crystal structure of *P. pyralis* luciferase has been determined by X-ray crystallography (Conti *et al*., 1996). Two different species of lampyrid genus, named *Lampyris turkestanicus* and *Lampyroidea maculata,* were reported from north of Iran (Geisthardt and Day, 2002). Recently, the complete sequence of cDNA encoding luciferase from *L. turkestanicus* (more available genus) has been reported (Alipour *et al*., 2004).

Although the structure-function relationship for the luminescence reaction has not been well resolved (Conti etal., 1996; Baldwin, 1996; Branchini *et al*., 2002), some amino acids are suggested to be important for catalysis (Viviani *et al*., 1999; Branchini *et al*., 2003; Branchini *et al*., 2005). However, one of the active site residues for the color determination (green to yellow) must be located in the fragment between 208 and 318 (Ohmiya *et al*., 1996).

Luciferase is widely used to determine small concentrations of ATP, because the enzymatic luminescence assay is highly sensitive, rapid, and nonradioactive and can be quantified in a

noninvasive manner (Gould and Subramani, 1988). Moreover, the cloning of the cDNA coding for firefly luciferase has opened a wide field of applications in molecular biology in which *luc* is used as a genetic marker or reporter gene (Wet *et al*., 1985). In fact, the luciferase gene has been utilized as a highly effective reporter gene in many organisms, such as amoeba (Baily and benard, 1994), bacteria (Jacobs *et al*., 1993), cellular slime moulds (Howard *et al*., 1988), plants (Miller *et al*., 1992), silkworms (Viskas *et al*., 1995) and mice (Dilella *et al*., 1988).

This paper reports the cloning, sequencing and expression of the cDNA for luciferase from *L. maculata*; this is a rare species from luciolinae subfamily, in Iran. Amino acid sequence similarity and homology modeling studies showed that the new luciferase belongs to the luciolinae subfamily and has a similar three dimensional structure to *P. pyralis* luciferase. We then included the new protein in a comparative sequence analysis and showed that this new luciferase contains some strictly conserved residues, which have been suggested to be important in functional properties. The sequence of *L. maculata* luciferase has been deposited in the GenBank as entry **DQ137139**.

Material and Methods

Firefly collection and extraction of total RNA. The firefly *L. maculata* was collected from the Amol forest, Mazandaran province, Northern Iran, and frozen in liquid nitrogen. The frozen fireflies were stored in a 80°C freezer. The lanterns of frozen firefly *L. maculata* (~0.5 g) were removed and pulverized under liquid nitrogen with a mortar and pestle. Total RNA was extracted using RNA extraction kit, according to the manufacturer's protocol (CinnaGen, Cat. No. RN7712C).

Reverse transcription-polymerase chain reaction. Total RNA was reverse-transcribed with the specific primer, and cDNA sequence encoding the luciferase was selectively amplified using specific primers. The primer sequences used for *L. maculata* were: 5'-ATGGAAATGGAAAAGGAGGAGAATGTTG-3' (sense primer), and 5'-TTACATCTTGGCTTGTGGTTTCTTAAGAA-3' (antisense primer) which was designed, based on the luciferase gene of *Luciola mingrelica* (GenBank™/EBI accession number S61961). The reverse transcription reaction was performed in a thermal cycler at 42°C for 60 min by 200 U/μL M-MuLV Reverse transcriptase (Fermentas, #EP0441), 20 U RNase inhibitor, dNTPmix (final concentration each at 1 mM), and reverse primer. The PCR reaction was carried out by 35 cycles of 94°C for 1 min, 57.8°C for 1 min and 72°C for 90 s and the final extension was performed at 72°C for 10 min. Amplified products were separated on 1% agarose gel, and ~1.7 kb long fragment were purified from the gel using the QIAquick gel extraction kit (Qiagen).

Cloning, subcloning and sequencing of *L. maculata* luciferase cDNA. Purified PCR products were cloned using InsTAclone PCR Cloning Kit (fermentas) which is a T/A based cloning system for direct one-step cloning of PCR-amplified DNA fragments with 3'-dA overhangs. DH5-α competent cells were transformed by the vector and plated on LB-Ampicillin plates treated with IPTG and X-GAL. White colonies were screened for *Luc*, by double digestion of isolated plasmid DNA and sequencing (MWG). Subsequently, *Luc* was amplified from positive colonies under the following conditions: initial denaturation, 94°C for 5 min; 28 cycles of amplification (94°C for 1 min, 60°C for 1 min, and 72°C for 90 s); final extension for 5 min at 72°C.

The cloning primers were 5'-CGAGTCGACAAATGGAAATGG AAAAGGAGGAG-3' for the translational start-sequence region and 5'-CGACTGCAGTTACATCTTGGCTTGTGGTTTC-3' for the 3' coding region and designed based on luciferase gene of *L. mingrelica* (GenBank™/EBI accession number S61961). The cDNA fragments were then digested by *Sal*I/*Pst*I and inserted into the digested/dephosphorylated pQE30. Competent cells of *E. coli* XL1-Blue (CinnaGen, Cat. No. BA7605C) were transformed by pQE30-Luc construct using electroporation.

Protein expression. Expression was achieved following a procedure similar to that of Devine *et al* (Devine *et al*., 1993). Bacteria containing the pQE30-Luc were grown overnight at 37°C on LB-Agar plates containing ampicillin and tetracycline. The master plates were sprayed with 200 μl solution containing of 0.2 mM D-luciferin (in 0.1 M Tris acetate) for 5 min and exposed to X-ray film for 4 h. After film development, the positive colonies (bioluminescent) were identified (they also could be observed by eye after dark-adaptation). Several positive colonies were picked from master plates, and the fresh LB-ampicillin/tetracycline medium was inoculated. The growth continued until the cell density reached an absorbance of ~0.5-0.6 (mid-log phase) at 600 nm (A_{600}), the culture was then induced with IPTG (isopropyl β-D-thiogalactoside, 0.1 mM) and the incubation was continued for an additional 16 h at 25°C. The bacterial cells were precipitated (5000 *g*, 20 min) and the pellet was suspended in lysis buffer (50 mM Na_2HPO_4, 0.3 M NaCl, 10mM imidazole, pH 8.0) and sonicated to disrupt the bacterial cells. The cell lysate was centrifuged and the supernatant was analyzed by SDS-PAGE.

Luciferase purification. The cell lysate was clarified by centrifugation (14,000 rpm, 25 min, and 4°C). The clarified lysate was applied to Ni-NTA Sepharose column and then washed with an imidazole step gradient (20, 40, 80, 120 and 250 mM imidazole in 50 mM NaH_2PO_4, 300 mM NaCl, pH 7.8). The histidine-tagged *L. maculata* luciferase was eluted with 120-250 mM imidazole, desalted using dialysis and placed into storage buffer (20% Glycerol). The purity of the luciferase was estimated more than 95% by SDS-PAGE.

Measurement of bioluminescence emission spectra. Bioluminescence emission spectrum of the luciferase from L. maculata was measured with a Perkin-Elmer luminescence spectrophotometer LS 50B apparatus. A volume of 50 μl of substrate mixture consisting of 1 mM luciferin, 50 mM Tris buffer, pH 7.8, 2 mM ATP and 10 mM $MgSO_4$ was added to 7 μl of purified luciferase solution in a quartz cell. Data were collected over the wavelength range 400-700 nm. The spectra were automatically corrected for the photosensitivity of the equipment.

Sequence analysis and homology modeling. The handling, analysis, and translation of the nucleotide sequences were performed with the tools available at the ExPASy Molecular Biology Server (www.expasy.ch). Pair wise and multiple sequence alignments were carried out using the BLAST and CLUSTAL W programs, respectively (Altschul *et al*., 1990; Karlin and Altschul, 1990). The structure of the *L. maculata* luciferase was modeled with the protein homology modeling SWISS-MODEL server using the crystal structure of Ppy luciferase (PDB code 1LCI) as template (swissmodel.expasy.org). Analysis and comparison of the structures were carried out using Swiss-PdbViewer ver3.7 (Guex and Peitsch, 1997). Phylogenetic analysis was performed using the Genebee service of the predicted amino acid sequences of known luciferase genes (www.genebee.msu.su).

Results

The cDNA encoding the luciferase from the lanterns of locally collected *L .maculata* was made by use of specific primers, expressed in *E. coli* and screened for functional production of light. The PCR product as determined on 1% agarose gel electrophoresis was about 1.7 kb which is sufficient to code for the entire luciferase polypeptide of approximately 550 amino-acid residues (data not shown).

Cloning, sequence analysis and characterization of the cDNA encoding *L. maculata*. To obtain a cDNA encoding the luciferase of the firefly *L. maculata*, a set of RT-PCR on the basis of the DNA sequence of *L. mingrelica* were designed (Devine *et al*., 1993). A cDNA fragment of approximately 1.7 kb was amplified, inserted into the T/A vector and sequenced.

The nucleotide sequence of insert was analyzed and its amino acid sequence was determined. The nucleotide sequence of the *L. maculata* cDNA and its deduced amino-acid sequence for the largest open reading frame has been deposited to gene bank. From the sequence analysis, the 1647 luciferase gene has an open reading frame of 548 amino acid residues.

The nucleotide and amino acid sequences were compared with those of known luciferase genes. The amino acid sequence of the luciferase gene of *L. maculata* showed 93% and 65% identity to *H. unmunsana* (GenBank™/EBI accession number **AN040975**) and *L. turkestanicus* (GenBank™/EBI accession number **AY742225**), respectively, while the lowest identity was found with *P. plagiophthalamus* (GenBank™/EBI accession number **AAQ11735**) (Table 1).

Phylogenetic analysis using gene and amino acid sequences data showed that *L. maculata* is in the same sister taxon of *H. unmunsana, Hotaria parvula,* and *L. mingrelica* within the Luciolinae subfamily (Fig. 1). Moreover, *L. maculata* allied with a clade of another species of Luciolinae namely; *L. lateralis* and *L. cruciata* (Fig. 1 and Table 1).

The crystal structure of *P. pyralis* was used as template to elucidate the structure of the *L. maculata* luciferase (Fig. 2). The sequence identity and the sequence similarity was 65% and 80%, respectively. Superposition of the 3-dimensional structures of *P. pyralis* and *L. maculata* luciferase revealed quite similar structures, and the predicted model was typically less than 1.95 RMSD from the crystal structure of template. Furthermore, similar to crystal structure of *P. pyralis* luciferase, *L. maculata* luciferase consists of a large N-terminal domain (approx. residues 1-436) and a small C-terminal domain (approx. residues 440-550) (Fig. 2).

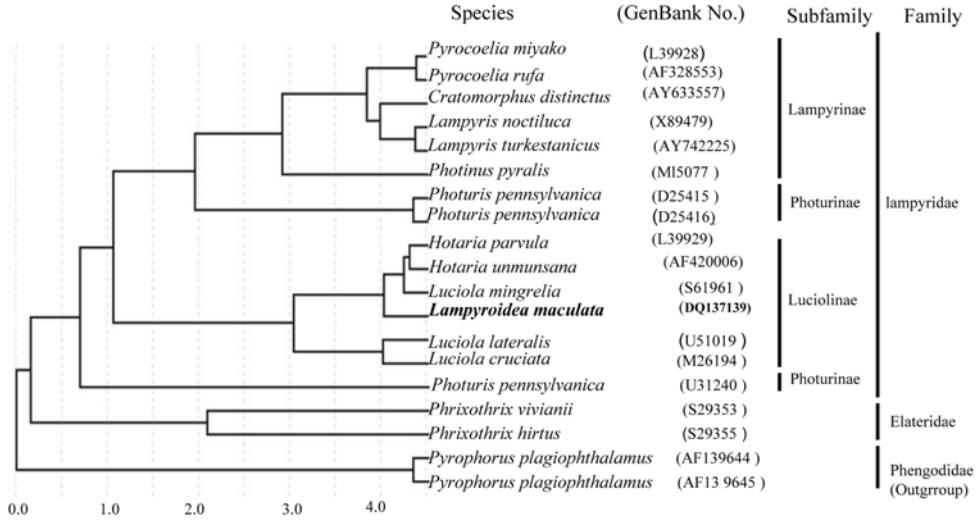

Fig. 1. Phylogenetic tree of firefly luciferases. A phylogenetic dendrogram (phylogram) was generated with Clustal-W alignment of known firefly luciferase sequences using the TreeTop program (www.genebee.msu.su/genebee.html). The branch lengths (*X* axis) in the rectangular cladogram represent the distances among those sequences calculated using BLOSUM62 substitution matrix.

Table 1. Pairwise comparisons among amino acid sequences of the *L. muculata* luciferase gene and the known luciferase genes

Species	GenBank Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1 *P. miyako*	L39928	-	0.012	0.081	0.058	0.056	0.167	0.259	0.257	0.332	0.333	0.324	0.333	0.316	0.324	0.351	0.411	0.450	0.458	0.458
2 *P. rufa*	AF328553	0.012	-	0.077	0.056	0.054	0.165	0.259	0.257	0.329	0.330	0.322	0.331	0.314	0.322	0.347	0.411	0.450	0.456	0.456
3 C. distinctus	AY633557	0.081	0.077	-	0.054	0.052	0.144	0.243	0.241	0.336	0.335	0.332	0.337	0.315	0.320	0.322	0.399	0.443	0.443	0.442
4 *L. noctiluca*	X89475	0.058	0.056	0.054	-	0.014	0.135	0.241	0.238	0.327	0.328	0.320	0.330	0.309	0.313	0.326	0.398	0.442	0.438	0.438
5 *L. turkestanicus*	AY742225	0.056	0.054	0.052	0.014	-	0.133	0.236	0.233	0.324	0.325	0.317	0.327	0.309	0.314	0.322	0.397	0.442	0.436	0.436
6 *P. pyralis*	M15077	0.167	0.165	0.144	0.135	0.133	-	0.259	0.257	0.326	0.323	0.321	0.332	0.316	0.316	0.347	0.386	0.433	0.450	0.450
7 *P. pennsylvanica*	D25415	0.259	0.259	0.243	0.241	0.236	0.259	-	0.017	0.375	0.371	0.374	0.378	0.360	0.363	0.369	0.413	0.454	0.440	0.439
8 *P. pennsylvanica*	D25416	0.257	0.257	0.241	0.238	0.233	0.257	0.017	-	0.373	0.369	0.372	0.377	0.359	0.361	0.371	0.414	0.453	0.439	0.438
9 *H. parvula*	L39929	0.332	0.329	0.336	0.327	0.324	0.326	0.375	0.373	-	0.021	0.022	0.050	0.152	0.156	0.414	0.432	0.469	0.483	0.485
10 *H. unmunsana*	AF420006	0.333	0.330	0.335	0.328	0.325	0.323	0.371	0.369	0.021	-	0.032	0.048	0.155	0.156	0.412	0.431	0.468	0.480	0.481
11 *L. mingrelica*	S61961	0.324	0.322	0.332	0.320	0.317	0.321	0.374	0.372	0.022	0.032	-	0.050	0.155	0.159	0.420	0.433	0.470	0.481	0.482
12 *l. maculata*	DQ137139	0.333	0.331	0.337	0.330	0.327	0.332	0.378	0.377	0.050	0.048	0.050	-	0.144	0.141	0.412	0.431	0.462	0.478	0.479
13 *L. lateralis*	U51019	0.316	0.314	0.315	0.309	0.309	0.316	0.360	0.359	0.152	0.155	0.155	0.144	-	0.051	0.406	0.439	0.471	0.479	0.479
14 *L. cruciata*	M26194	0.324	0.322	0.320	0.313	0.314	0.316	0.363	0.361	0.156	0.156	0.159	0.141	0.051	-	0.405	0.426	0.465	0.471	0.471
15 *P. pennsylvanica*	U31240	0.351	0.347	0.322	0.326	0.322	0.347	0.369	0.371	0.414	0.412	0.420	0.412	0.406	0.405	-	0.420	0.457	0.440	0.439
16 *P. viviannii*	AF139644	0.411	0.411	0.399	0.398	0.397	0.386	0.413	0.414	0.432	0.431	0.433	0.431	0.439	0.426	0.420	-	0.244	0.444	0.441
17 *P. hirtus*	AF139655	0.450	0.450	0.443	0.442	0.442	0.433	0.454	0.453	0.469	0.468	0.470	0.462	0.471	0.465	0.457	0.244	-	0.475	0.471
18 *P. plagiophthalamus*	s39353	0.458	0.456	0.443	0.438	0.436	0.450	0.440	0.439	0.483	0.480	0.481	0.478	0.479	0.471	0.440	0.444	0.475	-	0.019
19 *P. plagiophthalamus*	s29355	0.458	0.456	0.442	0.438	0.436	0.450	0.439	0.438	0.485	0.481	0.482	0.479	0.479	0.471	0.439	0.441	0.471	0.019	-

Numbers above the diagonal are mean distance values; numbers below the diagonal are absolute distance values.

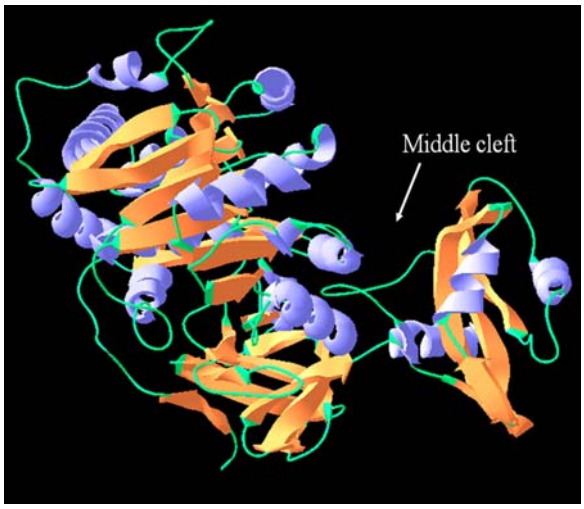

Fig. 2. Structural modeling of *L. maculata* luciferase. The ribbon presentation of structural models were built using SWISS-MODEL server, based on crystal structure of *P. pyralis* (Protein Data Bank code: 1lci). The α-helices are in blue, β-sheets in *orange,* and the turns are in green. The middle cleft is the putative active site region, where some conserved residues are located.

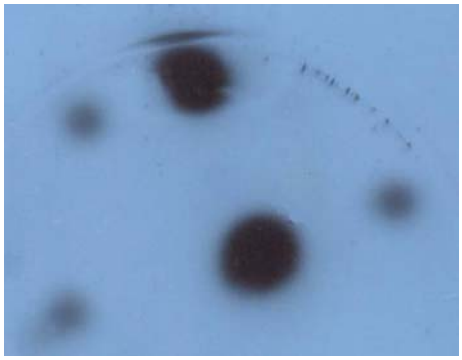

Fig. 3. The glowing colonies expressing luciferase were detected by exposure to X-ray film. Each spot shows a glowing colony with luciferase containing plasmid. For further details please see Material and methods.

Expression of *L. maculata* luciferase in *E. coli* XL1-Blue. The DNA fragment was then sub-cloned into the pQE30 plasmid to generate recombinate *E. coli* expressing luciferase. pQE30-Luc was constructed by digestion of pQE30 with *Sal*I and *Pst*I, followed by ligation of *L. maculata* luciferase gene. *E. coli* XL1-Blue was transformed by recombinant vector pQE30-Luc using electroporation. To verify the luciferase expression in *E. coli* cells, the transformed cells were assayed for luciferase activity by checking of light emission in the dark, using an X-ray film (Fig. 3). The expression of luciferase gene by recombinant XL1-Blue cells was analyzed by SDS-PAGE (data not shown). The expressed luciferase was appeared as a band of about 61 kDa. The cDNA encoding luciferase containing a polyhistidine tag (6x His) at the amino terminus of the protein was used to express luciferase. Cells were lysed and the clarified cytoplasmic extracts applied on a column. The polyhistidine-tagged luciferase was finally eluted from the column by increasing the imidazole concentration gradient up to 250 mM. The SDS-PAGE analysis of the eluted fractions showed that the polyhistidine-tagged enzymes were efficiently bound to the column and that the corresponding fractions contained highly purified protein.

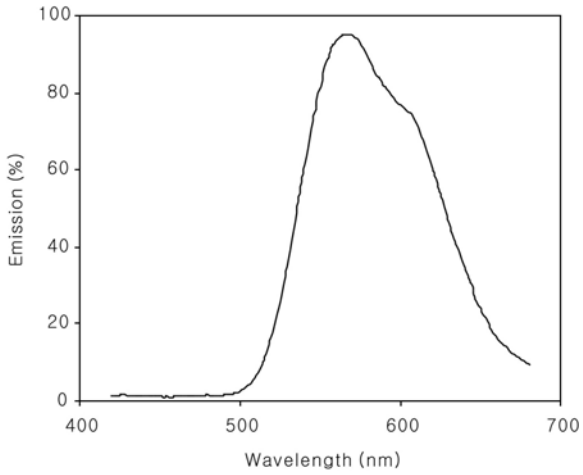

Fig. 4. Emission spectra of *L. maculata* luciferase was measured with a spectrofluorimeter. Emission spectra were measured by adding 7 µl of purified luciferase solution into 50 µl of substrate mixture.

Bioluminescence emission spectra. The color of the light emitted by *L. maculata* luciferase was green as that of the firefly. Fig. 4 shows the bioluminescence emission spectra of this luciferase in presence of luciferin and ATP at pH 7.8.

Discussion

In this study, the first cDNA encoding the luciferase from lantern mRNA of *Lampyroidea maculata* has been cloned, sequenced and expressed functionally in *E. coli*. The complete sequence of this cDNA is comprised of 1647 bp encoding a luciferase of 548 amino acid residue. The amino acid sequence analysis showed that the *L. maculata* luciferase is highly similar to *H. unmunsana* luciferase (GenBank™/EBI accession number AN040975) which indicated 93% identity in amino-acid sequences.

The phylogenetic analysis of the *L. maculata* luciferase gene with that of other light-emitting beetles showed relatively low relationship among the species of luciolinae, including *H. parvula, H. unmunsana, L. mingrelica, L. lateralis, L. cruciata,* forming two hetrophyletic groups. However, the *L. maculata* luciferase belongs to the same clade of *H. unmunsana, H. parvula,* and *L. mingrelica* species. Furthermore, the phylogenetic analysis among luciferase genes from several

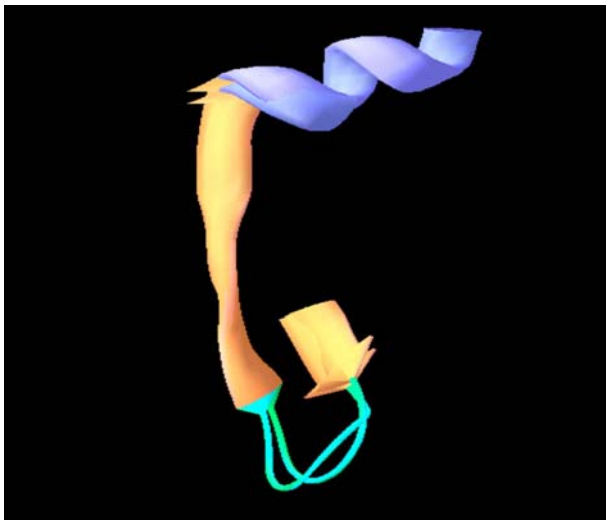

Fig. 5. Alignment of the *L. maculata* ATP-binding domain (residues 197-215) with corresponding nucleotide-binding domain in 4-coumarate: CoA ligase (residues 189-204). The *colorings* of the secondary structures of segments are the same as shown in Fig 4.

firefly species confirms phylogenetic utility of the gene for taxonomic purposes.

Related sequence and domain structure. The deduced amino acid sequence of the cDNA from *L. maculata* was compared with those of proteins in the NCBI and PIR data banks. Although *L. maculata* only shares 35% similarity to 4-coumarate: CoA ligase (GenBank™/EBI accession number **AY792353**), as revealed by blast analysis, putative amino acid sequence located in the fragments 197-215 and 435-466 in *L. maculata* corresponding to the fragments 189-204 and 431-462 in 4-coumarate: CoA ligase are 75% and 78% identical, respectively. Furthermore structural model of a fragment between 197-215 in *L. maculata* and its corresponding sequence in 4-coumarate: CoA ligase was similar, with only minor deviation in the connecting loop (Fig. 5). This seems to confirm that these residues have an important similar functional role in AMP binding between these two enzymes. Moreover, this may be of paramount importance in specificity of protein-nucleotide interactions and can provide an excellent model to investigate structure-function relationships.

Using ProSite (Gattiker *et al*., 2002), a domain structure map for the predicted amino acid sequence of *L. maculata* luciferase was developed. Table 2 defines the sites, different motifs, and their functions in *L. maculata* luciferase. It was reported (Gould *et al*., 1989; De Hoop. and Ab., 1992; Keller and Subramani, 1988) that, in some peroxisomal proteins, the targeting signal (PTS) resides in the last three amino acids of the C-terminus. In most firefly, glow-worm and all road-worm luciferases, SKL is known as a consensus sequence of targeting signal (Wood K. V., 1995). However in *L. maculata* like other species of luciolinae the microbody targeting tripeptide is AKM.

Table 2. Different motifs and sites in *Lampyroidea maculata* luciferase

Amino acid position	Motif information
116 NHTL 119 199 NSSQ 202 298 NKSE 301 310 NLTE 313	ASN-GLYCOSYLATION (N-glycosylation site)
48 TGVD 51 53 SYQE 56 111 TLRE 114 268 TKFD 271 278 TMQD 281 494 VPDP 497	CK2-PHOSPHO-SITE (Casein kinase II phosphorylation site)
546 AKM 548	Microbodies C-terminal targeting signal
49 GVDISY 54 96 GLYIGV 101 102 GVAPTN 107 160 GGYDCV 165 205 GLPKGV 210 250 GMFTTL 255 318 GAPLAK 323 337 GVRQGY 342 343 GLTETT 348	N-myristoylation site
61 SCR 63 111 TLR 113 129 SSK 131 181 SFK 183 364 SGK 366 380 TKK 382 496 TEK 498 529 TGK 531	PKC-PHOSPHO-SITE (Protein kinase C phosphorylation site)
197 IMNSSGSTGLPK 208	Putative AMP-binding domain signature

Sequence features and structural modeling. Multiple sequence alignment of firefly luciferases revealed that conserved residues are spread along the whole sequence but N-terminal regions are the least conserved sequences. The C-terminus in *L. maculata* luciferase shows the same key characteristics as those of *H. unmunsana* and *L. mingrelica*. There are a few important substitutions in *L. maculata* gene compared with *H. unmunsana* and *L. mingrelica* in a region with major effect on luciferase activity (Sala-Newby and Campbell, 1991; Waud *et al*., 1996). Amongst them, the substitution of Gly216 in *H. unmunsana* and *L. mingerlica,* in a region which is proposed for protein thermostability (Tatsumi *et al*., 1992) with Ala in *L. maculata* is noteworthy.

The solving of the crystal structure of *P. pyralis* luciferase without bound substrates or ligands revealed an apparently unique molecular architecture consisting of two distinct domains (Conti *et al*., 1996). However, a detailed description of the luciferase active site remains elusive. Additional mutational studies (Branchini *et al*., 1998, 2002, 2005) have identified individual amino acids and regions of the protein,

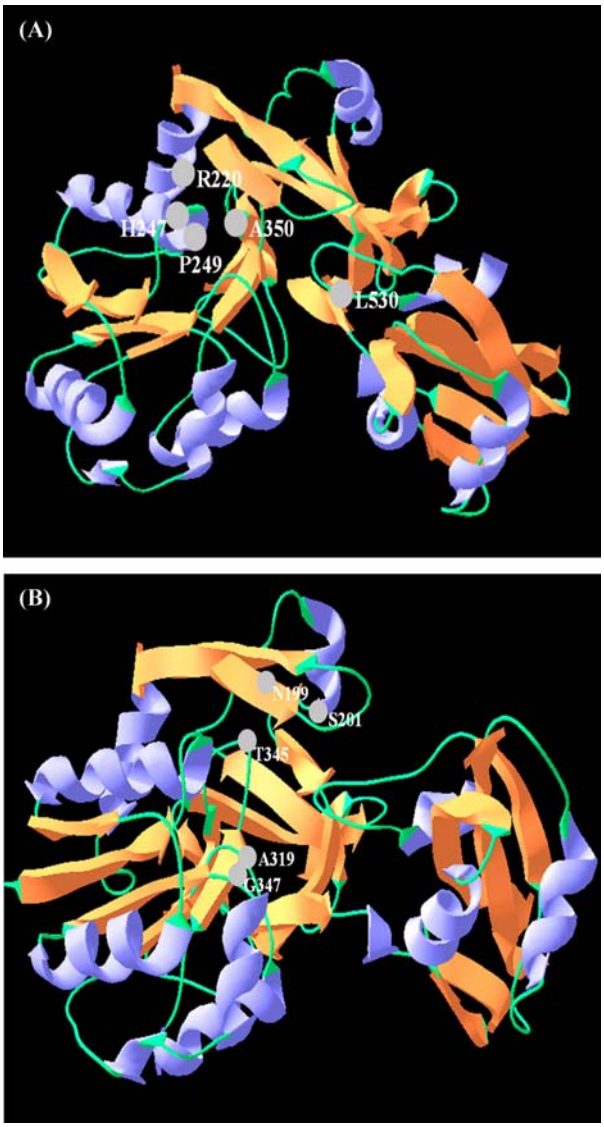

Fig. 6. Structural model of residues 195-535 in *L. maculata* luciferase. The invariant residues appear to interact with luciferin in putative luciferase binding site. These residues are Arg220, His247, Phe249, Ala350, and Lys531 in *L. maculata* luciferase that correspond to residues Arg218, His245, Phe247, Ala348, and Lys529 in Ppy lucifersae(A). Putative interacting residues with ATP in firefly luciferases. These residues are Asn199, Ser201, Thr345, Ala319, Gly341 in *L. maculata* luciferase that correspond to residues Asn197, Ser199, Thr343, Ala317, Gly339 in Ppy luciferase (B). Conserved residues in *L. maculata* are shown and in both figures, residues were found on the surfaces of the two Luc domains opposite to each other and separated by a wide solvent cleft.

the modification of which alters the color of bioluminescence. According to these studies a cavity is in a surface groove close to several invariant residues that comprise the putative luciferase active site. The crystal structure of Ppy luciferase showed that invariant residues Arg218, His245, Phe247, Ala348, and Lys529 appear to interact with luciferin in luciferase binding site (Branchini *et al*., 1998; Viviani *et al*., 1999). Furthermore the residues Asn197, Ser199, Thr343, Tyr340, Ala317, and Gly339 in Ppy luciferase sequence are supposed to interact with ATP (Hirokawa *et al*., 2002). Therefore, it can be concluded that residues Asn197 and Ser199 have a key role in ATP binding in *L. maculata,* as revealed by motif scan (Table 2).

Superposition of the three-dimensional model of *L. maculata* luciferase and three dimensional structure of *Photinus pyralis* luciferase reveals that the spatial arrangements of Luciferin- and ATP-binding residues are very similar (Fig. 6). According to this superposition, some residues appear to interact with luciferin are Arg220, His247, Phe249, Ala350, and Lys531 in *L. maculata* luciferase (Fig. 6A). In the other hand, Putative interacting residues with ATP are Asn199, Ser201, Thr345, Ala319, Gly341 in *L. maculata* luciferase (Fig. 6B). Conserved residues in *L. maculata* are shown and in both figures, residues were found on the surfaces of the two Luc domains opposite to each other and separated by a wide solvent cleft.

It has been suggested that the striking similarity in the spatial arrangement of these residues and their surroundings, along with adjacent residues may explain similar binding and kinetic properties. However, the similarity in the light emission spectrum of *L. maculata* (Fig. 4) to *P. pyralis* luciferase (not shown) suggests similarities in critical residues involving in formation of substrate intermediate structures. Moreover, modeling studies of *L. maculata* luciferase indicate specific residues in both putative binding sites are found on the surfaces of the two Luc domains, opposite to each other and separated by a wide solvent cleft.

Acknowledgment This work was supported by a grant from high-tech Department, Ministry of Mines and Industries (Iran) and research council of Tarbiat Modares University. We thank Ms. Z. Fazl-Zarandi for her helpful cooperation.

References

Alipour, B. S., Hosseinkhani, S., Nikkhah, M., Naderi-Manesh, H., Chaichi, M. J. and Kazempour Osaloo, S. (2004) Molecular cloning, sequence analysis and expression of a cDNA encoding the luciferase from the glow-worm, *Lampyris turkestanicus*. *Biochem. Biophys. Research. Comm*. **325**, 215-222.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.

Bailey, J., Benard, M. and Burland, T. G. (1994) A luciferase expression system for physarum facilitates analysis of regulatory elements. *Curr. Genet.* **26**, 126-131.

Baldwin, T. O. (1996) Firefly luciferase: The structure is known, but the mystery remains. *Structure* **4**, 223-228.

Branchini, B. R., Magyar, R. M., Murtiashaw, S. A. and Zimmer, M. (1998) Site- Directed Mutagenesis of Histidine 245 in Firefly Luciferase: A proposed model for the active site. *Biochemistry* **37**, 15311-15319.

Branchini, B. R., Murtiashaw, M. H., Magyar, R. A., Portier, N.

C., Ruggiero, M. and Stroh, J. (2002) The influence of the region between residues 220 and 344 and beyond in Phrixotrix railroad worm luciferases green and red bioluminescence. *J. Am. Chem. Soc.* **124**, 2112-2113.

Branchini, B. R., Southworth, T, L., Murtiashaw, M. H., Boije, H. and Fleet, S. E. (2003) A mutagenesis study of the putative luciferin binding site residues of firefly luciferase. *Biochemistry* **42**, 10429-10436.

Branchini, B. R., Southworth, T. L., Khattak, N. F., Michelini, E. and Roda, A. (2005) Red- and green-emitting firefly luciferase mutants for bioluminescent reporter applications. *Anal. Biochem.* **345**, 140-148.

Conti, E., Franks, N. P. and Brick, P. (1996) Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes. *Structure* **4**, 287-298.

De Hoop, M. J. and Ab, G. (1992) Import of proteins into peroxisomes and other microbodies. *Biochem. J.* **286**, 657-669.

DeLuca, M. (1976) Firefly luciferase. *Adv. Enzymol.* **44**, 37-68.

Devine, J. H., Kutuzova, G. D., Green, V. A., Ugarova, N. N. and Baldwin, T. O. (1993) Luciferase from the east European firefly *Luciola mingrelica*: cloning and nucleotide sequence of the cDNA, overexpression in *Escherichia coli* and purification of the enzyme. *Biochem. Biophys. Acta.* **1173**, 121-132.

De Wet, J. R., Wood, K. V., Helinski, D. R. and DeLuca, M. (1985) Firefly luciferase gene: structure and expression in mammalian cells. *Proc. Natl. Acad. Sci. USA* **82**, 7870-7873.

Dilella, A. G., Hope, D. A., Chen, H., Trumbauer, M., Schwartz, R. J. and smith, R. G. (1988) Utility of firefly luciferase as a reporter gene for promoter activity in transgenic mice. *Nucleic Acids Res.* **16**, 4159.

Gattiker, A., Gasteiger, E. and Bairoch, A. (2002) ScanProsite: a reference implementation of a PROSITE scanning tool. *Appl. Bioinformatics* **1**, 107-108.

Gesthardt, M. and Day, J. C. (2002) *Lampyroidea maculata*, coleopteran:*Lampyrida*.: a new species of lampyrid from Iran. *Zootaxa* **427**, 1-6.

Gould, S. J., Keller, G.-A., Hosken, N., Wilkinson, J. and Subramani, S. J. (1989) A conserved tripeptide sorts proteins to peroxisomes. *Cell Biol.* **108**, 1657-1664.

Gould, S. J. and Subramani, S. (1988) Firefly luciferase as a tool in molecular and cell biology. *Anal. Biochem.* **175**, 5-13.

Guex, N. and Peitsch, M. C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* **18**, 2714-2723.

Hirokawa, K., Kajiyama, N. and Murakami, S. (2002) Improved practical usefulness of firefly luciferase by gene chimerization and random mutagenesis. *Biochim. Biophys. Acta* **1597**, 271-279.

Howard, P. K., Ahern, K. G. and Firtel, R. A. (1988) Establishment of a transient expression system for Dictyostelium discoideum. *Nucleic Acids Res.* **16**, 2613-2623.

Jacobs, W. R., Barletta, R. G., Udani, R., Chan, J., Kalkut, G., Sosne, G., Kieser, S. T., Hatfull, G. F. and Bloom, B. R. (1993) Rapid assessment of drug susceptibilities of mycobacterium tuberculosis by means of luciferase reporter phages. *Science* **260**, 819-822.

Karlin, S. and Altschul, S. F. (1990) Methods for assessing the statistical significance of molecular sequence features by using general scoring schemes. *Proc. Natl. Acad. Sci. USA* **87**, 2264-2268.

Keller, G. A. and Subramani, S. (1988) Identification of peroxisomal targeting signals located at the carboxy terminus of four peroxisomal proteins. *J. Cell Biol.* **107**, 897-905.

McElroy, W. D., Seliger, H. H. and White, E. H. (1969) Spectral emission and quantum yield of firefly bioluminescence. *Photochem. Photobiol.* **10**, 153-170.

Miller, A. J., Short, S. R., Chua, N. H. and Say, S. A. (1992) A novel circadian phenotype based on firefly luciferase expression in transgenic plants. *Plant Cell* **4**, 1075-1087.

Ohmiya, Y., Ohmiya, Hirano, T. and Ohashi, M. (1996) The structural origin of the color differences in the bioluminescence of firefly luciferase. *FEBS Lett.* **384**, 83-86.

Sala-Newby, G. B. and Campbell A. K. (1991) Engineering a bioluminescent indicator for cyclic AMP-dependent protein kinase. *Biochem. J.* **279**, 727-732.

Tatsumi, H., Kajiyama, N. and Nakano, E. (1992) Molecular cloning and expression in *Escherichia coli* of a cDNA clone encoding luciferase of a firefly, *Luciola lateralis*. *Biochim. Biophys. Acta.* **1131**, 161-165.

Viskas, B. P., Sumathy, S. and Karumathil, P. G. (1995) Baculovirus mediated high-level expression of luciferase in silkworm cells and larvae. *Biotechniques* **19**, 97-104.

Viviani, V. R., Arnoldi, F. G. C., Brochetto-Braga, M. and Ohmiya, Y. (2004) Cloning and characterization of the cDNA for the Brazilian *Cratomorphus distinctus* larval firefly luciferase: similarities with European *Lampyris noctiluca* and Asiatic Pyrocoelia luciferases. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* **139**, 151-156.

Viviani, V. R., Bechara, E. J. H. and Ohmiya, Y. (1999) Cloning, sequence analysis, and expression of active *phrixothrix* railroad-worms luciferases: Relationship between bioluminescence spectra and primary structures. *Biochemistry* **38**, 8271-8279.

Waud, J. P., Sala-Newby, G. B., Matthews, S. B. and Campbell, A. K. (1996) Engineering the C-terminus of firefly luciferase as an indicator of covalent modification of proteins. *Biochim. Biophys. Acta.* **1292**, 89-98.

White, E. H., Rapaport, E., Seliger, H. H. and Hopkins, T. A. (1971) The chemi- and bioluminescence of firefly luciferin: an efficient chemical production of electronically excited states. *Bioorg. Chem.* **1**, 92-122.

Wood, K. V. (1995) The chemical mechanism and evolutionary development of beetle bioluminescence. *Photochem. Photobiol.* **62**, 662-673.