

Opiate Analgesics' Dual Role in Firefly Luciferase Activity

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ABSTRACT: The effects of three opiate analgesics, isolated from opium, on the firefly luciferase enzyme have been studied. Morphine (MN), 6-acetylmorphine (MAM), and diacetylmorphine (DAM) inhibited the enzyme activity at different levels. At lower concentrations, MN and MAM enhanced enzyme activity, effecting inhibition at higher concentrations. However, DAM inhibited the enzyme activity at all concentrations investigated. The stimulating activity of MN and MAM is attributed to the hydrophilic interaction of the proton donor–acceptor type with the polar regions of the luciferase located outside the binding pocket of the active site. The inhibition at higher concentrations of MN and MAM and at all concentrations of DAM is found to be competitive in nature, with the analgesics competing for the binding of the enzyme's natural substrate luciferin. The binding site of the luciferase could accommodate only one analgesic molecule. Binding constants determined from bioluminescence studies showed that the inhibitor binding site is hydrophobic in nature. The inhibition constants of analgesics are in the order MN > MAM > DAM. The greater binding of DAM to luciferase is attributed to its ability to form a ground state complex with ATP and greater hydrophobicity. At higher concentrations of ATP, the binding constants increased. The results obtained are explained assuming that the firefly luciferase acts as a subtype μ -opioid receptor model.

Opiates are known for their prototypical analgesic and pharmacological importance (1, 2). It is observed that opiates inhibit either the basal (3) or neurostimulated increase in the adenylate cyclase activity in several mammals (4–7) and cause respiratory depression (8) due to their action on the brain stem respiratory centers. Owing to their immense importance in biochemical studies, the concept of opioid receptor was first postulated by Beckett and Casy (9), and subsequently, Portoghesi and co-workers (10) proposed different opiate receptors for structurally different opioids. In more recent years, through extensive in vivo and in vitro research, there is now considerable support for the existence of three major opioid receptors, i.e., μ -, δ -, and κ -opioid receptors, all of which belongs to the G-protein family (11).

Morphine and its alkaloid analogues are considered the prototypical μ -receptor agonists (12, 13). The μ -opioid receptor and firefly luciferase enzyme have similar catalytic sites. *N*-Ethylmaleimide modification studies of luciferase (14) and μ -opioid receptors (15–17) revealed that there are two vicinal sulfhydryl groups present near or in the active site of these proteins. The two sulfhydryl groups in both the proteins, located on a single polypeptide chain, yield disulfide bonds and upon modification of these thiol groups by reagents result in decreased enzymatic activity. Firefly luciferase is the best characterized among the bioluminescent enzymes, and the light produced in this system is directly related to the ATP present in the cells; its high sensitivity

led to the ultrasensitive assay for the determination of ATP present in a variety of biomolecules in biology and medicine (18). Franks and Lieb (19) exploited this efficient bioluminescent enzyme system and established that general anesthetics act directly on proteins with amphiphilic target sites. Moreover, the traditional view is that anesthetics relieve pain by rendering the person unconscious while the narcotic analgesics deaden the pain by raising the threshold to pain and diminishing the alarm reaction to sustained pain (20). In view of the structural similarities existing between luciferase and μ -opioid receptor and also adhering to the statement that at this time there is no gold standard for defining the opioid receptor selectivity (21), since most tissues express several classes of receptors leading to one type of ligand inhibiting the activities of other types of receptors (22–25), we have investigated the protein analgesic interaction by following bioluminescence (BL¹), emission, and electronic absorption spectroscopy. The protein considered in this study is firefly luciferase, and the opiates used are morphine (MN), 6-acetylmorphine (MAM), and diacetylmorphine (DAM).

MATERIALS AND METHODS

Materials

The opium sample was obtained from Government Opium and Alkaloid Factories (Ghazipur, India). Firefly D-luciferin

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¹ Abbreviations: MN, morphine; MAM, 6-acetylmorphine; DAM, diacetylmorphine (heroin); BL, bioluminescence; A, analgesic; ATP, adenosine triphosphate.

Table 1: Physical and Spectral Data of Opium Analgesics

	morphine	6-acetylmorphine	3,6-diacetylmorphine
mp ^a (°C)	245–248	265–270	235–237
UV-vis (in C ₂ H ₅ OH) λ_{\max} (nm)	288	299, 308 (weak)	240, 281, 311
IR (KBr, cm ⁻¹)	3480, 3400, 3210, 2940, 2920, 2840, 1605, 1650, 1090, 1250, 760	3400, 2940, 1740, 1630, 1605, 1205	2940, 1765, 1735, 1600, 1250, 1180
NMR ^b (column 1, DMSO- <i>d</i> ₆ ; CDCl ₃ /DMSO- <i>d</i> ₆ , column 3)	6.76 (d), 6.68 (d), 5.75 (d), 5.40 (d), 5.06 (d), 4.37 (m), 4.20 (d), 3.0 (s), 2.16	—	2.37, 2.11 (s), 2.90 (s), 3.35 (m), 3.80 (m), 5.55 (d), 5.40 (m), 5.80 (d), 6.90 (d), 6.70 (d)

^a Melting points are uncorrected. ^b s, singlet; d, doublet; m, multiplet.

and luciferase (LDR) were purchased from Amersham as the RPN 1630 bioluminescent ATP assay kit. Stock solutions were made in the buffer supplied with the kit and frozen at -80°C as aliquots. ATP was obtained from Pharmacia, and the stock was made in 10 mM Tris-HCl at pH 7.5. The working stock solutions were freshly prepared on the day of use. The BL assay was performed at $25 \pm 1^{\circ}\text{C}$ after the rapid mixing of luciferase, luciferin, and ATP·Mg²⁺ with or without analgesic using an external mixer. For all the experiments, the final concentration of luciferase was on the order of 10 nM in glycylglycine buffer at pH 7.8. Variable concentrations of luciferin, ATP, and analgesics were used. The reaction was initiated by the injection of ATP, and the final reaction volume was always kept at 400 μL .

Methods

The bioluminescence light output was measured using the LKB 1250 luminometer connected to a recorder. All the readings were taken 5 s after mixing. The maximum activity was taken as the peak of the light output. The absorption spectra were obtained by using a Hitachi 150-20 UV-vis spectrophotometer. Corrected fluorescence spectra were recorded on a Hitachi 4010 spectrofluorimeter by maintaining the excitation and emission bandwidths at 5 nm. The IR spectra were obtained using Perkin-Elmer 283B spectrometer. NMR data were obtained from the Bruker WH300 spectrometer. HPLC analysis was performed on a Waters 626S pump system using a reverse phase C-18 Hewlett-Packard 200 \times 4.6 mm, 5 μm HPLC column. The elution buffer was 70% 15 mM phosphate buffer (pH 3.5) with 30% acetonitrile. The flow rate was 1 mL/min. The eluate was monitored at 230 nm by employing a Linear 206 PHD detector.

Extraction and Preparation of Analgesics. Morphine was extracted from opium powder in the presence of lime. The filtrate was collected, and crude MN was precipitated by the addition of ammonium chloride. Pure MN sulfate was obtained by dissolving the crude in dilute H₂SO₄ and precipitating with ammonium hydroxide. DAM was prepared by refluxing MN (25 mmol) with distilled acetyl chloride (50 mmol, BDH) under an alkaline pH. The mixture was then dissolved in water and neutralized with sodium carbonate. Crude DAM that precipitated out was filtered and recrystallized twice in acetone. MAM was obtained by refluxing MN (20 mmol) with distilled acetic anhydride (25 mmol, E-Merck). Mono- and diacetylmorphine were converted into their respective chloride salts by the addition of

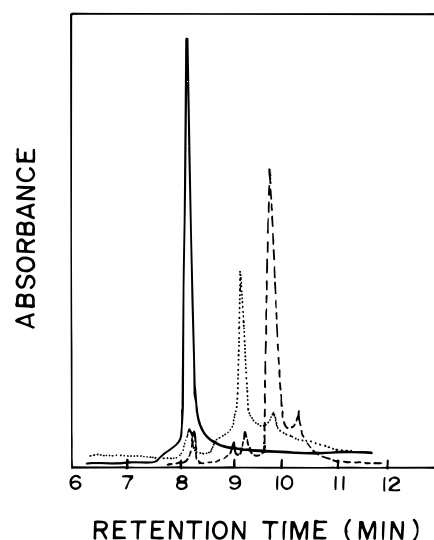


FIGURE 1: HPLC purity of analgesics: (—) MN, (···) MAM, and (---) DAM.

calculated amounts of HCl during recrystallization (26). The melting points of the compounds prepared were in agreement with the reported values. The electronic absorption maxima, IR, and NMR data of the three opiates are shown in Table 1. The stocks of these opiates were prepared in quartz-distilled water and stored at 20°C . The purity of the analgesics was checked by HPLC (27) and was above 95% in all cases (Figure 1).

Inhibition Studies. The reciprocals of the peak height divided by the enzyme concentration were plotted against the reciprocals of luciferin concentrations in the absence or presence of opiate analgesics to obtain K_m (or K_m^{app}) and V_{\max} following a simple binding model (28). The average peak heights for the above double-reciprocal plots were obtained by performing the assay in triplicate. All the experiments were carried out in triplicate in order to fit the data into a simple linear regression analysis, instead of a weighted least-squares analysis (29). Inhibition constants, K_i , and the number of opiate analgesic molecules, N , involved in the inhibition were determined from the relationship $f(A) = (1 + [A]/K_i)$, where the $f(A)$ is the factor by which K_m^{app} for the competing luciferin changes with analgesic concentration. Plots of $f(A)$ or $\sqrt{f(A)}$ against the analgesic concentration were obtained to determine the inhibition constants and the stoichiometries of the analgesic molecules (30). The analgesic EC_{50} values are related to the dissociation constant, K_i , and were obtained using the relationship $\text{EC}_{50} = 2K_i$.

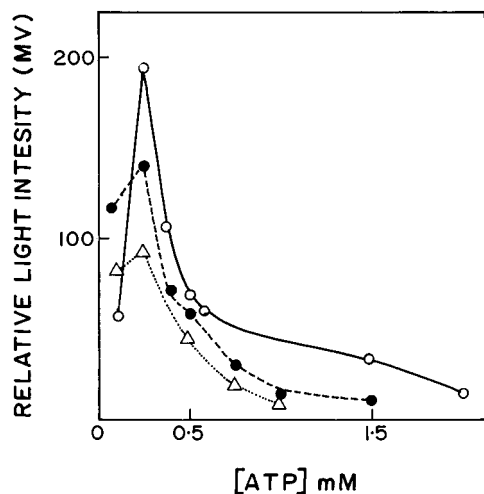


FIGURE 2: Bioluminescence of the luciferase system: 10 nM luciferase and (●) 1.5×10^{-5} M, (○) 3×10^{-5} M, and (△) 7.5×10^{-6} M luciferin.

RESULTS

Firefly Luciferase Inhibition by ATP

The relative intensities of light produced against varied concentrations of ATP at a fixed luciferin concentration are shown in Figure 2. The concentrations of luciferin and ATP employed throughout the investigation varied between 7.5–60 μ M and 0.125–1.5 mM, respectively. We found that the enzyme activity reached a maximum when the ATP concentration was 0.25 mM, and with a further increase in the concentration of ATP, the peak intensity decreased. The mechanism of inhibition of enzyme activity over a range of saturating levels of ATP concentrations was determined by measuring luciferase activity with varying luciferin concentrations between 7.5 and 60 μ M. We found that K_m , i.e., $K_m(\text{luciferin})$ or $K_m(\text{ATP})$, remains constant while the V_{\max} decreases with increasing ATP or luciferin concentration. It is observed that, with an increase in the ATP concentration in the range of 0.125–1.5 mM, the value of $K_m(\text{luciferin})$ remained constant with a value of 2.1 (SE of ± 0.11) $\times 10^{-5}$ M while the V_{\max} decreased from 3.5×10^{-6} to 1.47×10^{-7} per 10^{-8} M of enzyme concentration.

Firefly Luciferase Inhibition and Stimulation by Analgesics

Bioluminescence Studies. The maximum rate of light production is extremely sensitive to the presence of analgesics. The influence of MN on the activity of luciferase is shown in Figure 3A. Under the conditions where the active site of luciferase is saturated with luciferin and ATP, the addition of MN has different effects at different concentrations with respect to reaching peak height and rate of decay. A similar observation is also found with MAM regarding reaching the peak height but not with DAM. We observed a significant increase in the luciferase activity at relatively low MN and MAM concentrations. This enhancement in activity is accompanied by an altered rate of luminescent decay following the increase in peak intensity. MN and MAM at higher concentrations and DAM at all concentrations (Figure 3B) inhibited the activity of the enzyme. In addition to the decrease in the peak intensity with increasing

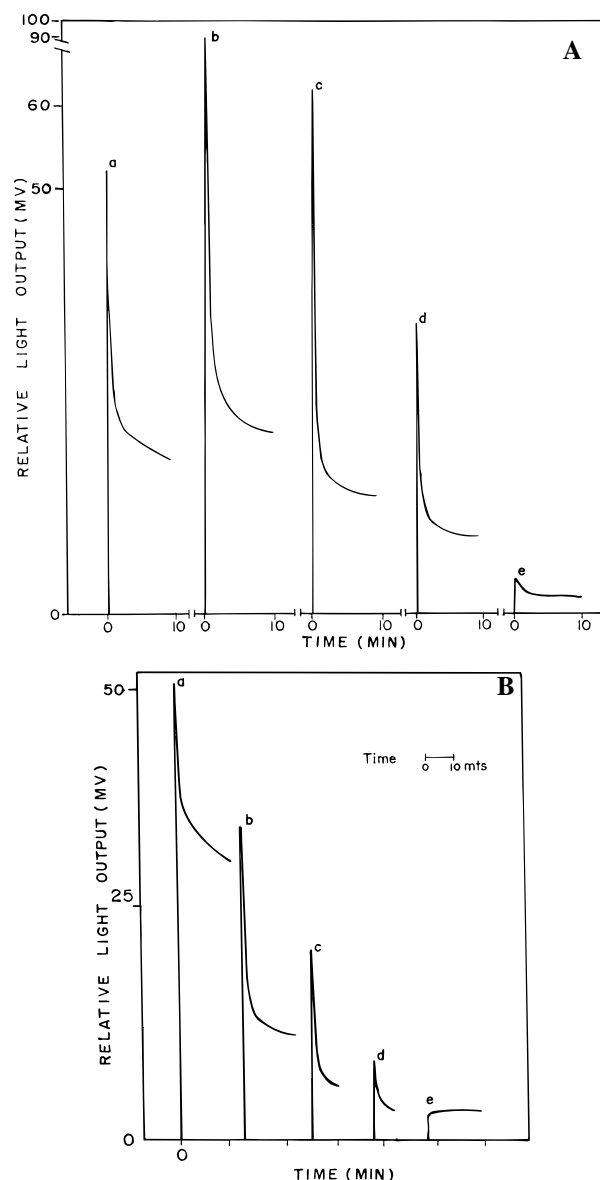


FIGURE 3: (A) Effect of MN on luciferase activity: luciferase (10 nM), luciferin (7.5 μ M), and ATP (0.25 mM) with (a) control, (b) 0.25 mM, (c) 0.50 mM, (d) 0.75 mM, and (e) 1.0 mM MN. (B) Effect of DAM on luciferase activity: luciferase (10 nM), luciferin (7.5 μ M), and ATP (0.25 mM) with (a) control, (b) 1.77×10^{-4} M, (c) 2.2×10^{-4} M, (d) 2.66×10^{-4} M, and (e) 3.54×10^{-4} M DAM.

analgesic concentration, a little increase in the time to reach the peak intensity together with a considerable decrease in the rate of decay of the luminescence following the peak height is also noticed. All the three analgesics investigated inhibit the luciferase activity by competing with luciferin for binding to the protein at all ATP concentrations. The mechanism of inhibition is determined by measuring luciferase activity over a range of luciferin concentrations in the absence and presence of inhibitor. In Figure 4, the double-reciprocal primary plot, i.e., the reciprocal of the maximum rate of light production (the peak height), is plotted against the reciprocal of the luciferin concentration at various concentrations of MN. Similar double-reciprocal plots are also obtained with MAM and DAM at a constant concentration of ATP. The series of lines shows that as the analgesic concentration increases the K_m^{app} increases without a sig-

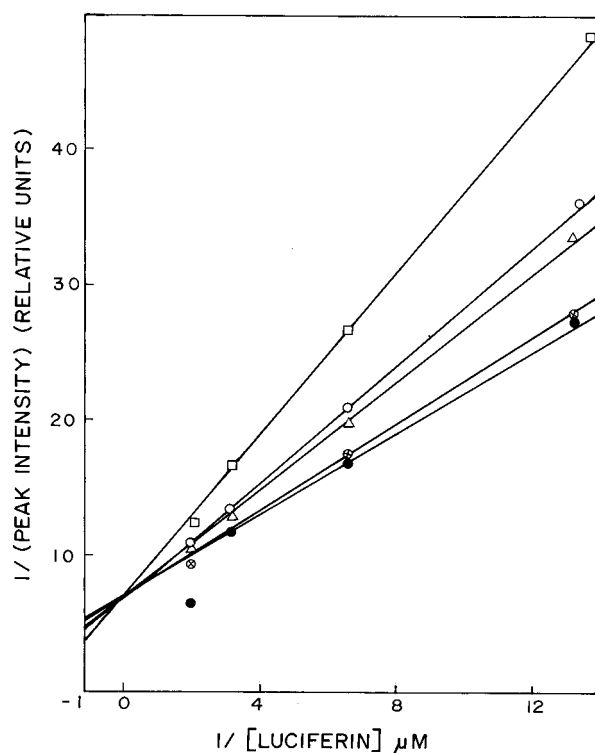


FIGURE 4: Double-reciprocal plots of the luciferase-luciferin, MN system: luciferase (10 nM) and ATP (0.5 mM) with (data points overlapping) (●) control, (⊗) 0.5×10^{-3} M, (△) 1.0×10^{-3} M, (○) 1.25×10^{-3} M, and (□) 5.0×10^{-3} M MN.

nificant change in the maximum activity (V_{\max}). Typical data showing $K_m^{\text{app}}/K_m(\text{luciferin})$ or $\sqrt{f(A)}$ against analgesic concentrations yielded the inhibition constant K_i . The EC_{50} values calculated theoretically from the equation $\text{EC}_{50} = 2K_i$ are given in Table 2, along with the inhibition constants. ATP occurs ubiquitously in living organisms and is the most important central coupling agent between exergonic and endergonic processes in all cells. It is either consumed or regenerated in every metabolic sequence. Further, the physiological ATP concentration present in all cells is varied. We considered worthwhile seeing the modulation of ATP on the analgesic-protein interaction and obtained the double-reciprocal plots at various ATP concentrations. The data obtained at various ATP concentrations are presented in Table 2. It is obvious from Table 2 that as the enzyme is saturated with increasing amounts of ATP the binding constants also increase, inferring that the binding constants are ATP-dependent and at all ATP concentrations only one analgesic binds to the enzyme.

Excited State Studies. To infer a possible mechanism for the stimulated luciferase activity at lower concentrations of MN and MAM, electronically excited and ground state interactions between analgesics and luciferin, analgesic and ATP, and analgesic and luciferase are studied. Denburg and co-workers (31) observed that oxyluciferin,² the product formed during luminescence, is a competitive inhibitor of the enzyme. If MN and MAM prevent the inhibition of enzyme by oxyluciferin, then they must react with the oxyluciferin or its structural analogue luciferin either in the

ground state or in the excited state. However, we found that all the analgesics experienced an interaction with the excited luciferin but not with ATP. Similarly, no interaction of analgesic with the enzyme was observed in the excited state. Given in Figure 5 is the evidence for the exciplex formation between the excited luciferin and the ground state analgesics (luciferin*—analgesic). The relative intensity of the luciferin emission band at 530 nm is quenched with the addition of analgesics having an isoemissive point at 485 nm without any change in the emission wavelength. The Stern-Volmer quenching constant (K_{sv} , the slope derived from a linear plot obtained from the relationship $F_0/F = 1 + K_{sv}[Q]$) for luciferin*—DAM is found to be 250 M^{-1} and for luciferin*—MN is 570 M^{-1} . These large K_{sv} values and isoemissive points are an indication of the efficient quenching of luciferin emission by analgesics and are evidence of exciplex formation between luciferin and DAM and luciferin and MN. Similar exciplex formation between the excited state analgesics and ground state luciferin is also observed. The absence of any significant change in fluorescence spectra of analgesics with the addition of ATP or vice versa indicates that there is no specific interaction between analgesic and ATP in the excited state. The fluorescence spectra of luciferase and luciferin with and without analgesics are shown in Figure 6. The emission spectrum of the aqueous mixture containing luciferase-luciferin and MN obtained by irradiating at luciferin's absorption maxima (335 nm) is unique in exhibiting dual emission, a weak emission at 433 nm along with a 537 nm emission band, whereas the aqueous solutions containing luciferase-luciferin and DAM or luciferase and luciferin show an emission maximum only at 537 nm.

Ground State Studies. Similarly, the electronic absorption spectra of luciferin-analgesic, ATP-analgesic, and luciferase-analgesic systems are studied for a possible ground state interactions between these systems. The electronic spectral studies reveal that no perceptible interaction exists between the luciferin-analgesic, ATP-MN, and ATP-MAM systems. However, from Figure 7, we inferred that a ground state interaction is present between ATP and DAM. The aqueous solution of DAM displays three absorption maxima at 305 nm (broad) and 282 nm and a shoulder at 240 nm, while ATP absorbs strongly at 259 nm. With the addition of increased amounts of ATP to DAM and also with the equilibrium concentration of ATP being kept in the reference path to cancel out the absorption of free ATP, the 282 nm transition of DAM experienced a blue shift, ultimately stabilizing at 248 nm. The equilibrium constant K , for the ground state complex formed between DAM and ATP, is determined by following the Baba-Suzuki (32) relationship, and it is found to be $6.25 \times 10^2 \text{ dm}^3 \text{ M}^{-1}$ ($\log \epsilon = 4.5$). The larger values are indicative of a greater affinity of ATP toward the DAM but not the MN or MAM, though they are structurally similar. The electronic absorption spectral studies for the enzyme-analgesic systems did not provide any useful information as they displayed overlapping electronic transitions. However, we noticed that, when either MN or MAM was added to luciferase, there is a considerable broadening of the absorption maxima of luciferase with the peak shifting more toward longer wavelengths with hyperchromism.

² Oxyluciferin is the light-emitting molecule in the bioluminescence reaction, and it is highly unstable. Hence, we have considered luciferin a model of oxyluciferin as they are structurally similar.

Table 2: Inhibition Constants and Theoretical Effective Concentrations at 50% Activity (EC_{50}) for Analgesics^a at Various ATP Concentrations

analgesic	1.25×10^{-4} M ATP		2.5×10^{-4} M ATP		5.0×10^{-4} M ATP		7.5×10^{-4} M ATP	
	$K_i \times 10^{-4}$ (M)	$EC_{50} \times 10^{-4}$	$K_i \times 10^{-4}$ (M)	$EC_{50} \times 10^{-4}$	$K_i \times 10^{-4}$ (M)	$EC_{50} \times 10^{-4}$	$K_i \times 10^{-4}$ (M)	$EC_{50} \times 10^{-4}$
MN	13.8	27.5	10.9	21.8	9.38	18.76	8.12	16.2
MAM	3.56	7.12	1.35	2.69	1.98	3.96	—	—
DAM	2.39	4.77	1.52	3.05	1.07	2.15	—	—

^a Only one analgesic molecule binds at the active site of the enzyme.

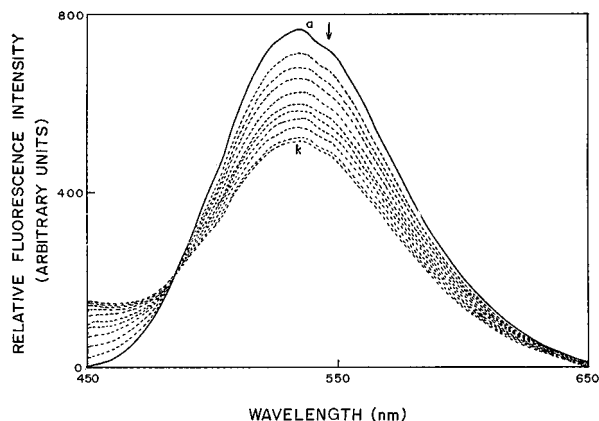


FIGURE 5: Emission spectra of luciferin-analgesics and various MN concentrations in H_2O , with λ_{ex} at 335 nm: (a) 7.5×10^{-6} M luciferin and (b–k) luciferin with 7.5×10^{-6} M and 7.4×10^{-5} M, 4.7×10^{-5} M, 22.0×10^{-5} M, 29.0×10^{-5} M, 36.1×10^{-5} M, 43.0×10^{-5} M, 50.9×10^{-5} M, 56.6×10^{-5} M, 63.2×10^{-5} M, and 70×10^{-5} M MN, respectively.

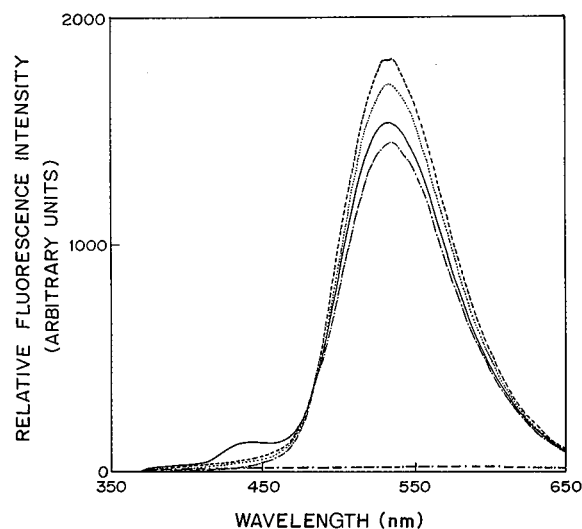


FIGURE 6: Emission spectra of luciferin and luciferase, with and without analgesics in H_2O , with λ_{ex} at 335 nm: (---) 12.5×10^{-6} M luciferin, (---) 2.2×10^{-7} M luciferase, (---) 12.5×10^{-6} M luciferin and 2.2×10^{-7} M luciferase, (---) 12.5×10^{-6} M luciferin, 2.2×10^{-7} M luciferase, and 0.25×10^{-3} M MN, and (---) 12.5×10^{-6} M luciferin, 2.2×10^{-7} M luciferase, and 0.1×10^{-3} M DAM.

DISCUSSION

Lee and co-workers (33) observed that ATP is a competitive inhibitor of luciferase. However, in our studies, it is revealed that excess ATP decreases the turnover number of the enzyme while effecting no considerable change in K_m (luciferin). Constancy in K_m and a decrease in V_{max} indicate that the mechanism of ATP inhibition is noncompetitive. It is, therefore, suggested that the nature of inhibition at higher and under ATP saturation conditions of the luciferase enzyme

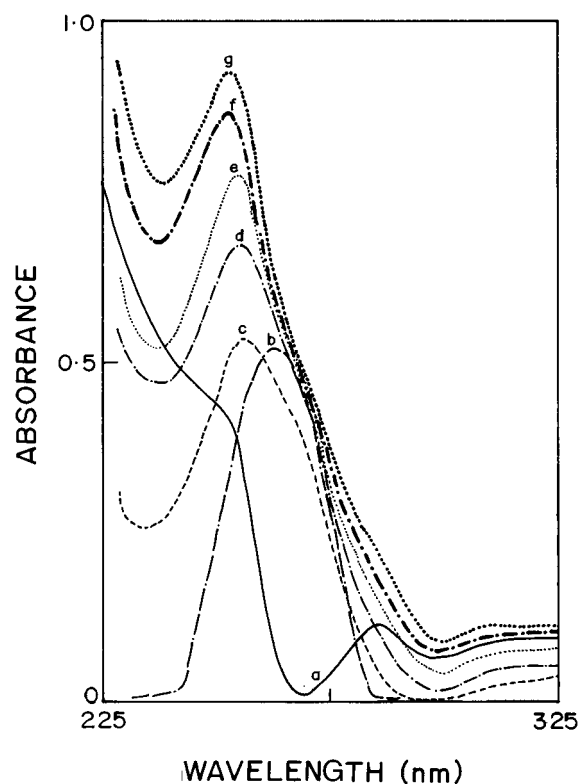
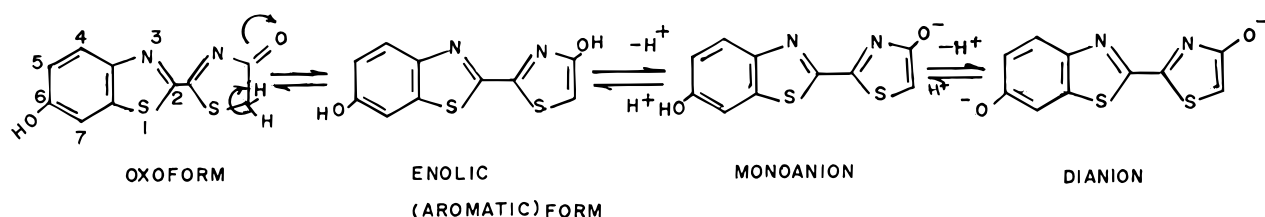


FIGURE 7: Absorption spectra of the ATP-DAM system in water: (a) 5.7×10^{-4} M DAM, (b) 4.2×10^{-5} M ATP, and (c–g) 5.7×10^{-4} M DAM and 4.2×10^{-5} , 8.5×10^{-5} , 13.0×10^{-5} , 17×10^{-5} , and 22×10^{-5} M ATP, respectively (reference path containing the equilibrium concentration of ATP).

is allosteric, i.e., excess ATP molecules not competing with the luciferin substrate at the substrate binding site, but noncompetitive inhibition via allosteric conformational change. Further, the constancy in the K_m values with reciprocal affinities of luciferin and ATP for the enzyme explains the random binding characteristics of these substrates in the order of their mixing (34).

Opioid-protein interaction investigations would be useful enough for developing therapeutic agents for the opioid receptors (4). Firefly luciferase, a protein, combines with its substrate luciferin in the presence of $ATP \cdot Mg^{2+}$ and O_2 to give a photon of light. The maximum rate of light production is extremely sensitive to the presence of analgesics at all concentrations of ATP that were employed. From Figure 4, it could be seen that as the dose of analgesics increases the K_m^{app} increased but has no significant effect on the maximum activity. Further information on the analgesic-protein interaction is obtained from the linear increase of $f(A)$ with an increase in analgesic and also fit a quadratic equation accurately, suggesting that only one analgesic molecule is involved in the competition at the active site of the enzyme. The enzyme inhibition abilities

Scheme 1



of the three analgesics studied are found in the order DAM > MAM > MN. The trend in inhibition could be explained by considering the hydrophobic (or nonpolar) nature of the binding site in the luciferase (35, 36). Since DAM does not possess any polar functional groups, it can be described as being relatively more hydrophobic than MAM which in turn is more hydrophobic than MN. Therefore, it is reasonable to observe higher binding constants for a relatively more hydrophobic analgesic compound. If analgesic and luciferin bind to the same site, one might envisage modulation by ATP of the analgesic sensitivity of the luciferase. This is in fact observed, though the variation is very little, a small increase in the inhibition constant as the ATP concentration decreased (Table 2). This sensitivity of firefly luciferase at high ATP concentrations may be attributed to the increased hydrophobicity of the analgesic–luciferin binding pocket with increasing ATP concentrations (19).

Inhibition by Analgesics. When the ground state interactions are studied using electronic absorption spectra, no direct interactions between luciferin and analgesic, ATP and MN, and ATP and MAM are observed, indicating that in the ground state no luciferin–analgesic interaction is responsible for either stimulation or inhibition of the enzyme activity. However, a strong complex formed between ATP and DAM with an equilibrium constant of $6.25 \times 10^2 \text{ dm}^3 \text{ M}^{-1}$ is an indication of the luciferase inhibition by DAM, which could now be attributed to the combined effect of two factors. First, because of the complex formation between each DAM and ATP, the availability of ATP at the active site is decreased in the presence of DAM or the ATP–DAM complex itself might diminish the enzyme activity, and second, its competition with luciferin for the binding site of the enzyme. Probably any stimulation brought by DAM is counteracted by its simultaneous inhibitory effects.

Further, the emission studies reveal that there is no interaction present between the ATP and the three analgesics at the excited state, but all the analgesics formed exciplexes with the luciferin. The large K_{sv} constants indicated that strong exciplexes formed between luciferin (or the oxyluciferin) and the analgesics, leading to the decreased light output due to the increased nonradiative decay of the excited oxyluciferin. However, the magnitudes of K_{sv} values suggest that MN should inhibit to a larger extent than DAM. It is therefore now proposed that in case of MN and MAM the reduced light output could be attributed to the increased nonradiative decay process of excited oxyluciferin due to exciplex formation and their competition with luciferin for binding to the protein. Whereas in the case of DAM in addition to the above two factors, its ability to form a ground state complex with ATP also might effect the enzyme inhibition.

Excitatory Effects of Analgesics. As mentioned earlier, DAM at all concentrations inhibited the enzyme activity while MN and MAM have dual effects on luciferase. At lower concentrations, MN and MAM stimulated the luciferase while at higher concentrations are involved in competitive inhibition with luciferin for the active site. Similar observations were made by Curry and co-workers (30) with ethanol, *n*-propanol, acetone, and chloroform producing excitation effects on luciferase and are attributed to interactions of these molecules with the small and relatively polar sites on the protein. However, there are no detailed molecular interpretations offered for these excitatory effects. Electronic spectral studies between luciferase and analgesics could not yield any quantitative estimations since both of them had overlapping absorption maxima. Nevertheless, it is worth noting that the broadening of the band at the 300 nm transition of luciferase, when MN and MAM

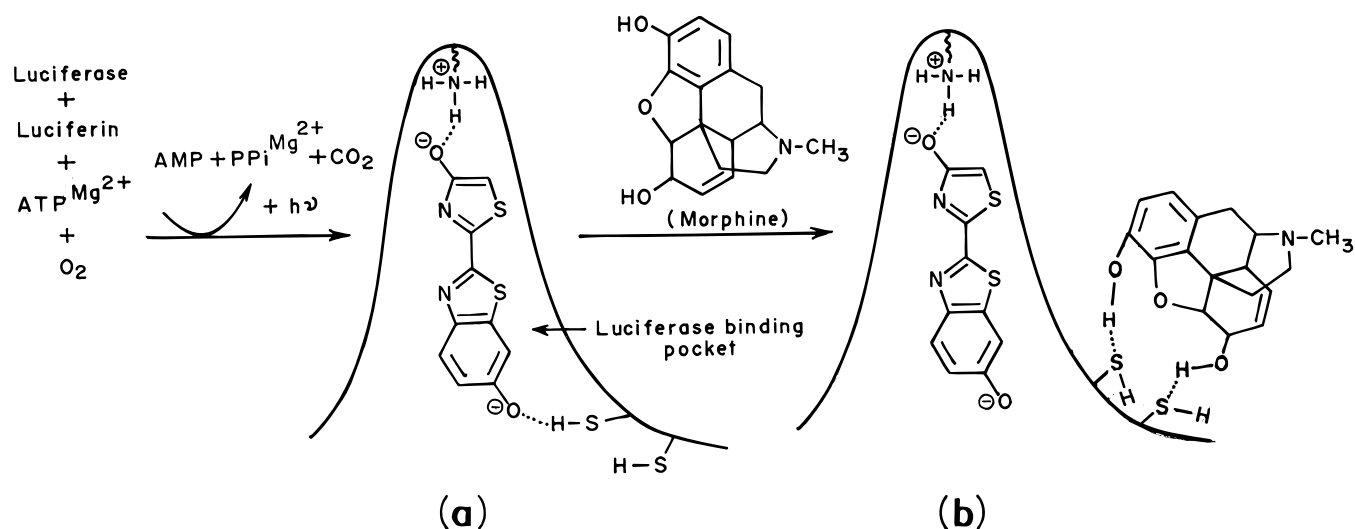


FIGURE 8: Tentative model for the enhanced luciferase activity (a) in the absence of morphine and (b) in the presence of morphine.

are mixed separately with luciferase in water shifting the absorption max toward red, indicates that the phenolic group in MN or MAM is forming H bonds with the polar sites, including those near the surface of the binding pocket of the protein, resulting in the enhancement of the enzyme activity at lower concentrations of these two analgesics (14). However, the more interesting revelation from fluorescence studies of enzyme and luciferin in the presence of analgesics is derived from Figure 6. The λ_{em} maxima observed at 433 ± 1 nm for luciferase, luciferin, and MN solution, when excited at 335 nm, could be attributed to the free phenolic 6-OH group of the luciferin or monoanionic oxyluciferin (37) (Scheme 1). When MN or MAM enters the binding site, its phenolic group forms H bonds with the polar sites at the surface of luciferase, setting one polar group of the substrate luciferin free. As the product is an enzyme-bound monoanion, its emission is shifted to lower wavelengths, i.e., 433 nm compared to the dianion emitting at 535 nm. This phenomenon might lead to a mechanism of increased enzyme activity at lower concentrations of MN and MAM.

Excitatory and Inhibitory Effects. On the basis of the above results, we explain the stimulatory effect of MN and MAM on the luciferase with two vicinal sulfhydryl groups located at the surface of the active site of the enzyme. The product formed during BL is a bifunctional molecule with one phenolic group; the other one is either oxo, or the oxo group is in a tautomeric form with the phenolic group in the thiazole moiety. This bifunctional molecule when present in the amphiphilic active site of the enzyme is held by two H bonds at different locations and also hydrophobic interactions in the binding pocket as shown in Figure 8a. The H bond that can be seen in the well of the hydrophobic site is between the amino group of the lysine which is present in the bottom of the active site's well and the phenolic group of luciferin or oxyluciferin. The other hydrogen bond is formed at the surface of the active site involving the thiol groups of luciferase and the 6-OH group of the product formed. The rate of release of the product from the active site is an important factor in an overall rate of enzymatic reaction. The release of product, however, may be viewed as the culmination of a series of steps taken in a regular pattern or random order. When MN or MAM is added in small amounts, the following may occur in series. The MN or MAM added may break H bonds present at the surface of the active site between sulfhydryl groups of the enzyme and the 6-OH group of the product, and the next step would be the immediate seizing of the boundary region by MN and MAM and forming H bonds with their acidic (phenolic OH) group with sulfhydryl groups of luciferase, earlier involved in the H bonding with the product. In the absence of associative interaction at the boundary, the product would be a hanging type and would dissociate itself from the active site and be expelled from the binding pocket faster than in the absence of MN or MAM, resulting in stimulation of luciferase as shown in Figure 8b. It is worth noting that, when the vicinal sulfhydryl groups at the surface of the binding pocket of the luciferase are modified by methylation or converted to disulfides, the activity of the enzyme is decreased (14, 16, 17). Whereas the same sulfhydryl groups increase the enzyme activity in the present case. This is probably due to increased electron density on sulfur because of its attachment to an electron-donating group like methyl,

while decreasing the electron density on sulfur by making a H bond with MN or MAM may activate the enzyme. The inhibition of the enzyme activity by DAM and at higher concentrations of MN and MAM is due to the competition of these analgesic molecules with the luciferin substrate for binding to the protein and ground and excited state complex formation between the analgesics and substrate. The protein-analgesic interactions presented in this paper further underline the major difficulties encountered in investigating the pharmacological specificity of opioid receptors.

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