Molecular Uniformity in Biological Catalyses

The enzymes concerned with firefly luciferin, amino acid, and fatty acid utilization are compared.

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Early studies, on the mechanism of energy transformation in microorganisms and skeletal muscle, revealed the necessity of coupling exergonic or "energy liberating" reactions to biosynthetic or functional processes. Sufficient data had accumulated by the 1940's to indicate that the chemical coupling agent in cellular energy transformation is the pyrophosphate bond of adenosine triphosphate (ATP) (*1*). This pyrophosphate bond energy was subsequently implicated in almost all biosynthetic processes requiring energy.

Biochemists have recently been concerned with the mechanisms of energy utilization in the synthesis of macromolecules such as complex polysaccharides, fats, nucleic acids, and protein. From these and earlier studies it soon became evident that the initial reaction in the utilization of many compounds having a carboxyl (COOH) group involves a unique step requiring ATP.

In 1941, Lipmann predicted that high-energy phosphate groups (~p) are essential for the incorporation of amino acids into protein (*1*). The nature of the initial step was first suggested by studies concerned with fatty acid metabolism in which it was demonstrated that the formation of acetyl coenzyme A in mammalian tissue involves a pyrophosphorolysis of ATP yielding adenylic acid (AMP) and inorganic pyrophosphate (PP) (*2*). In the same year, Maas and Novelli discovered that, in the synthesis of pantothenic acid from pantoic acid and β-alanine, stoichiometric amounts of AMP and PP appear (*3*). A similar observation concerning the synthesis of hippuric acid from benzoic acid and glycine was also reported (*4*). Both syntheses involve the formation of a peptide bond (*5*). By 1954 the primary mechanism for the activation of carboxyl groups which were involved in biosynthetic reactions was suggested to be as follows (*6*):

$$RCOO^- + ATP \overset{Mg^{++}}{\leftrightarrows} R\overset{O}{\overset{\|}{C}} - AMP + PP \quad (1)$$

Hoagland presented direct evidence that reaction 1 is the general mechanism for amino acid activation (*7*). Berg reported similar data for acetate activation and suggested that acetyl-AMP is the intermediate compound leading to the formation of acetyl coenzyme A (*8*). Peng (*9*) and Jencks and Lipmann (*10*) reported similar mechanisms for the activation of longer-chain fatty acids. In 1958, Rhodes and McElroy indicated that a similar activation step is necessary for light emission in firefly extracts and demonstrated the formation of dehydroluciferyl-adenylate (reaction 6) (*11*). At the same time several investigators reported the formation of tryptophanyl-adenylate by a purified enzyme (*12*).

By 1958, there was convincing evidence that the initial step in the utilization of fatty acids, amino acids, luciferin, pantoic acid, and benzoic acid involved the formation of an acyl-adenylate and pyrophosphate (*5*). The general equation for activation and subsequent transfer may be written

$$R\overset{O}{\overset{\|}{C}}O^- + ATP \overset{Mg^{++}}{\rightleftarrows} R\overset{O}{\overset{\|}{C}} - AMP + PP \quad (2)$$

$$R\overset{O}{\overset{\|}{C}} - AMP + X \rightleftarrows R\overset{O}{\overset{\|}{C}} - X + AMP \quad (3)$$

We now know that the acceptor, X, may be coenzyme A in the case of acetate and dehydroluciferin activation, transfer RNA (tRNA) for amino acid activation, or oxygen for luciferin activation. It has been difficult to demonstrate reaction 2 because the acyl-adenylates are tightly bound to the enzyme and therefore do not accumulate. In a few cases when large amounts of enzyme were used, it has been possible to isolate equivalent amounts of acyl-adenylates (*13–17*).

Sufficient evidence has been obtained on the mechanism of action of these synthetases to make it worth while to compare their properties (*18*). An understanding of the mechanism of formation and reaction of acyl-adenylates and the physical and chemical properties of the corresponding synthetases should help in judging the relative importance of the enzymes in initiating and regulating synthetic processes.

Because of our more immediate interest and knowledge of the mechanism of action of luciferase we present its properties in detail and compare, where possible, the characteristics of this enzyme with fatty acid acyl coenzyme A synthetase and aminoacyl tRNA synthetase.

The reactions catalyzed by luciferase are as follows:

$$LH_2 + ATP + E \overset{Mg^{++}}{\rightleftarrows} E \cdot LH_2\text{-}AMP + PP \quad (4)$$

$$E \cdot LH_2\text{-}AMP + O_2 \rightarrow \text{light} + \text{products} \quad (5)$$

$$L + ATP + E \overset{Mg^{++}}{\rightleftarrows} E \cdot L\text{-}AMP + PP \quad (6)$$

$$E \cdot L\text{-}AMP + CoA \rightleftarrows E + L\text{-}CoA + AMP \quad (7)$$

where LH_2 is luciferin, L is dehydroluciferin (Fig. 1), and E is enzyme.

The reactions catalyzed by fatty acyl coenzyme A synthetases are

$$FA + ATP + E \overset{Mg^{++}}{\rightleftarrows} E \cdot FA\text{-}AMP + PP \quad (8)$$

$$E \cdot FA\text{-}AMP + CoA \leftrightarrows E \cdot FA\text{-}CoA + AMP \quad (9)$$

where FA is fatty acid.

The reactions catalyzed by aminoacyl-tRNA synthetase are

$$AA + ATP + E \overset{Mg^{++}}{\rightleftarrows} E \cdot AA\text{-}AMP + PP \quad (10)$$

$$E \cdot AA\text{-}AMP + tRNA \rightleftarrows E + AA\text{-}tRNA + AMP \quad (11)$$

where AA is amino acid. All of these enzymes have many properties in com-

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mon, the most important of which may be summarized as follows:

1) All synthetases catalyze an exchange between pyrophosphate and ATP, which is dependent upon the specific substrate (reactions 4, 6, 8, and 10). This indicates that pyrophosphate is a product of the activating step and that the reaction is reversible. The only possible exceptions thus far reported are the arginyl and glutamyl-tRNA synthetases (*19*). With these enzymes, pyrophosphate will exchange into ATP only if tRNA is added to the reaction mixture. Apparently the enzyme-tRNA complex is the active form of the enzyme that catalyzes the formation of the acyl-adenylate and of pyrophosphate.

2) The enzyme-bound acyl-adenylates [E·R(CO)-AMP] react under appropriate conditions with hydroxylamine forming the corresponding hydroxamic acid derivative. This suggests the presence of an activated carboxyl group.

3) In a few cases the intermediate of the activating reaction (acyl-adenylate) has been isolated. These acyl-adenylates are tightly bound to the enzyme, and their reactivity is usually restricted to those compounds next in the biosynthetic sequence.

4) The synthetases catalyze the transfer of the acyl-adenylate to an appropriate acceptor with the liberation of adenylic acid (reactions 7, 9, and 11). It is evident that AMP exchange depends upon the presence of the acceptor molecule. The AMP exchange indicates the reversibility of this transfer reaction. The only possible exceptions to the reversibility of this reaction involve the activation of lipoic acid and biotin. In these two reactions the bound acyl-adenylates react directly with a specific lysine residue in the corresponding apoenzyme, forming a stable amide bond and free AMP.

5) The activating step is specific for ATP and has an absolute metal requirement, namely, for Mg^{++} or Mn^{++} ions. In the case of luciferase and fatty acyl coenzyme A synthetases metals are not required in the transfer reaction (*8, 16, 20*). In the case of the transfer of aminoacyl-adenylates to tRNA there remains some difference of opinion concerning a metal requirement (*14, 15*).

6) As a general rule all of the enzymes require two or more sulfhydryl groups for activity. Sulfhydryl oxidation has been suggested to explain the instability of nearly all synthetases isolated. The one exception to this generality is the lysyl-tRNA synthetase from *Escherichia coli* (*21*). This enzyme has been studied extensively and has proved unusual in its insensitivity to sulfhydryl reagents. Interestingly enough, the lack of inhibition of the lysyl-tRNA synthetase from *E. coli* may be unique, since a similar activity in rat liver and ascites tumor can be inhibited by mercurials (*21*).

7) The evidence shows that only one acyl-adenylate is formed per enzyme molecule. The common structure for the binding of the acyl-adenylate to the enzyme is R(CO)OPO-adenosine. At least two groups must be recognized by the enzyme (the R and the adenine groups) although the acyl phosphate must contribute greatly to the tight binding of the acyl-adenylate to the enzyme. As discussed later, an acceptor molecule, in order to react with the enzyme-bound acyl-adenylate, must be capable of recognizing a specific configuration of the intermediate enzyme complex.

8) All synthetases (*16, 22–25*) except the acetyl coenzyme A synthetase (*26*) and the phenylalanyl-tRNA synthetase (*22, 27, 28*) have molecular weights near 100,000. The latter is reported to have a molecular weight between 150,000 and 180,000, which may reflect a basic difference in the protein structure of this enzyme from other synthetases or an aggregation such as that observed for the acetyl coenzyme A synthetase. The subunit structure of *Photinus pyralis* luciferase is composed of two subunits of molecular weight 51,000 (*24*). Apparently methionyl-tRNA synthetase also has subunit structure (*29*). The amino acid composition of four synthetases so far examined is similar (*23, 30, 31*).

Let us now consider the reactivity and specificity of the enzyme-bound acyl-adenylates. The hypothesis which we propose for all synthetases is that large conformational changes occur when specific substrates combine with these enzymes. These conformational changes alter the specificity and reactivity of the enzyme-bound acyl-adenylates.

Reactivity and Properties of Enzyme-Bound Acyl-Adenylates

Acyl-adenylates in aqueous solution are highly reactive. An indication of their reactivity is given by their lability

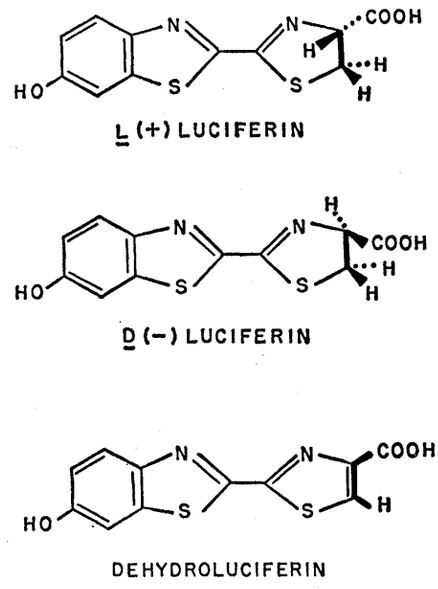

Fig. 1. Structure of luciferin and dehydroluciferin; D(—) luciferin is the natural form of luciferin.

at different *p*H's. At neutral or alkaline *p*H the acyl-adenylates are rapidly hydrolyzed to the corresponding free acids (*20, 32–36*). The extreme reactivity of the free acyl-adenylates is shown by studies of model systems. At neutral *p*H, acyl-adenylates react rapidly with hydroxylamine, thiols, alcohols, amines, imidazole, and water, releasing AMP and the respective acyl derivatives (*32*). Low concentrations of imidazole increase the rate of reaction with glutathione and coenzyme A. Acetyl-adenylic acid in the presence of imidazole forms a ribose-acetylated AMP in addition to acetylimidazole. This nonenzymic reaction is analogous to the transfer of amino acids to the 3-hydroxyl of the terminal AMP of tRNA. It might be supposed that a histidine of the enzyme near the binding site of the acyl-adenylates could act in a similar manner to catalyze the transfer of the acyl group to the appropriate acceptor. Evidence implicating such a histidine is still very limited.

In many cases, the enzyme-bound acyl-adenylate may be rapidly hydrolyzed, but the interaction with other molecules is greatly restricted. The nature of the binding of the acyl-adenylate to the enzyme appears to limit the accessibility of nucleophiles and thereby confers a specificity on the overall reaction.

In view of the reactivity of acyl-adenylates and the variety of nucleophilic groups in these enzymes, it is somewhat surprising that many non-

specific transfers do not occur. This must be attributed to the very tight and restrictive binding of the acyl-adenylate to the synthetases and may have significant biological implications with regard to specificity and regulation of biosynthetic processes involving acyl-adenylates.

Another important aspect of the enzyme-catalyzed reactions of acyl-adenylates concerns the equilibrium of the overall reaction. The equilibrium constant for the valyl-tRNA synthetase reaction at *p*H 7 is 0.32 (*35*). The constants for the threonyl-tRNA synthetase (*36*), acetyl coenzyme A synthetase (*37*), and the fatty acyl-coenzyme A synthetase of the intermediate chain are all about 1 at *p*H 7.0 (*38*). Zachau and Feldman have discussed the chemistry of acyl-adenylates and their derivatives (*34*).

In the case of the aminoacyl-tRNA synthetases, the rate of formation of aminoacyl-adenylate is 20 to 140 times faster than the transfer to tRNA (*35*); the opposite is true with luciferase where the oxidative step is ten times faster than formation of luciferyl-adenylate (*20*). It would be interesting to know if transfer from synthetic aminoacyl-adenylates to tRNA occurs at the same rate as the overall reaction.

The turnover number for the synthetases is unusually low. The acetyl coenzyme A synthetase has one of the highest turnover numbers, 910 moles of acetate per mole of enzyme per minute (*39*).

Reaction of Firefly Luciferase

Luciferyl-AMP and oxygen. Like the fatty acyl coenzyme A and aminoacyl-tRNA synthetases to be discussed below, firefly luciferase catalyzes the formation of luciferyl-adenylate (LH_2-AMP) from luciferin (LH_2) (Fig. 1) and ATP (*11*, *20*) (reaction 4). The enzyme-bound LH_2-AMP reacts rapidly with oxygen, giving rise to an excited-state intermediate which subsequently emits light (reaction 5 and Fig. 2). Although the nature of the intermediate and the product of the light reaction are unknown, one light quantum is emitted for each luciferin molecule oxidized, and one molecule of oxygen (O_2) is used in the process (*40*). The synthetic acyl-adenylate (LH_2-AMP) will react also with the enzyme to give light emission, thus eliminating the requirement for ATP.

Effect of enzyme and substrate structure on color of light emission. Light emission in an enzyme-catalyzed reaction depends upon the creation of an excited state in an enzyme-intermediate complex. The chromophoric group (oxidized LH_2-AMP) bound to the enzyme has been considered of primary importance in specifying the electronic transitions allowed, thus determining the color of light emitted. However, it has been demonstrated that both the structure and conformation of the enzyme are important factors in influencing the color of light. For example, analyses of more than 20 different species of fireflies indicate that, while all the luciferins are identical, the color of light emitted varies by over 600 angstroms (yellow-green to orange). Measurements in vitro of the light produced by individual firefly species show conclusively that the source of the enzyme is the primary factor which determines the color of light, suggesting structural differences in the luciferase of each species (*41*).

After these observations were made, it was possible to demonstrate that various environmental factors that affect the secondary and tertiary structure of luciferase would also alter the color of light. High temperature, for example, led to a red emission (617 mμ) compared to a normal yellow-green (562 mμ) for extracts of *Photinus pyralis*. In addition the color of the bioluminescence in vitro is markedly dependent upon the *p*H of the reaction solution (*42*). There are two different emitters (Fig. 3), one leading to the normal yellow-green light at neutral and alkaline *p*H, identical with that observed in the intact firefly, and the second yielding a red emission at acid *p*H (617 mμ). Urea, Zn^{2+}, Cd^{2+}, or Hg^{2+} ions also change the yellow-green light to red. From the foregoing it was concluded that configurational changes occur and alter the interaction of the enzyme with the excited intermediate, yielding a different emitting species.

One might also expect to change the color of light by altering the structure of the substrates (LH_2 and ATP) in a manner which would affect their bind-

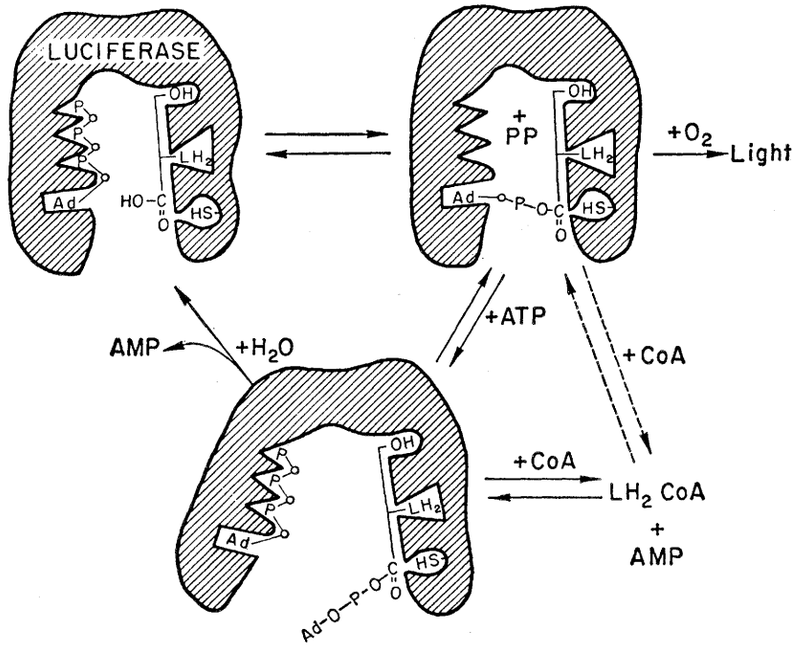

Fig. 2. Representation of luciferase action. There are at least two binding sites on luciferase, one for ATP and the second for luciferin. Both the adenine and the polyphosphate structure in ATP are important for binding. The hydroxyl and the carboxyl groups are important in the binding of luciferin. The initial reaction is the formation of luciferyl-adenylate (LH_2-AMP) and inorganic pyrophosphate. The enzyme-bound LH_2-AMP reacts rapidly with oxygen to give light and unknown products. Dehydroluciferin combines at the luciferin site and reacts with ATP forming dehydroluciferyl-adenylate (L-AMP). No light is emitted from this reaction. Enzyme-bound LH_2-AMP or L-AMP reacts readily with coenzyme A forming the corresponding thiol ester. The evidence indicates that the ATP binds at its normal active site as indicated in the figure, thus altering the reactivity of the bound acyl-adenylate (homosterism). In the absence of coenzyme A, water reacts rapidly with the acyl-adenylate–ATP-enzyme complex to hydrolyze the acyl-adenylate. If pyrophosphate is removed, there is therefore a continuous breakdown of ATP into AMP and PP.

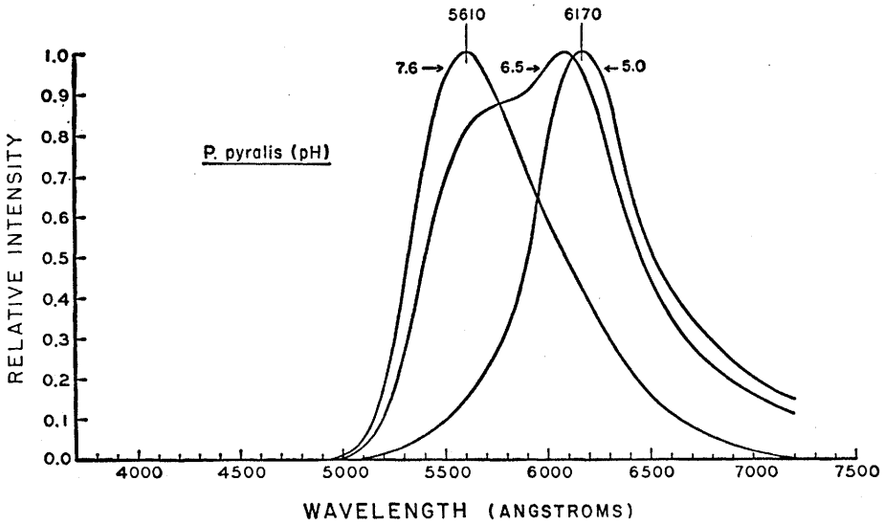

Fig. 3. Effect of *p*H on the bioluminescence emission in vitro of *Photinus pyralis* luciferase plus *P. pyralis* luciferin (Fig. 1). Reaction initiated with ATP and Mg^{2+} ions in 0.025*M* glycylglycine buffer. The spectra are normalized to 1 at the peak of emission.

ing to the enzymes. Unfortunately, it is not possible to change greatly the luciferin structure and still obtain an active light-emitting substrate; however, some luciferin analogs have been synthesized (*43*). As indicated in Fig. 4, aminoluciferin ($6'NH_2$-LH_2) gives a red emission instead of the yellow-green observed with natural luciferin. Furthermore, it is important to note that *p*H changes have no effect on the color of light emitted from the amino luciferin. This lack of effect and other data indicate that the ionization of the 6-hydroxyl group in luciferin is important in determining the nature of the emitter. By studying the appearance of the red emission at different *p*H's, one obtains a *pK* (6.8) that suggests that the hydroxyl group is interacting with a histidine residue. Apparently it is the phenolate ion which interacts and gives the normal light color; substitution with an amino group prevents this interaction and the *p*H effect.

Leonard and associates (*44*) have demonstrated that an ATP isomer with the ribose attached to the 3-position of the adenine ring is 10 to 15 percent as effective in the light reaction as normal ATP. The additional interesting observation is that, with the isomer, red light is emitted at *p*H 7.0, in comparison to the normal yellow-green observed with natural ATP. Even with the isomer it is possible to obtain a predominately yellow-green emission if the *p*H is shifted to 8.0. This suggests that the nucleotide attachment to luciferin and presumably to the enzyme is important in determining the color of the light.

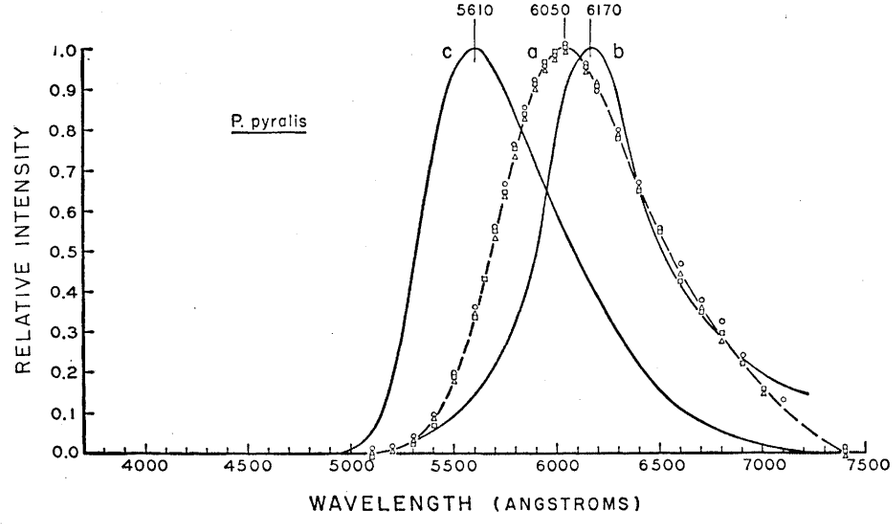

Fig. 4. Bioluminescence emission spectra of (*a*) *Photinus pyralis* luciferase plus 6'-amino luciferin at *p*H 6.0 (○), *p*H 7.7 (□), and *p*H 8.55 (△); (*b*) *P. pyralis* luciferase plus luciferin at *p*H 6.0, and (*c*) *P. pyralis* luciferase plus luciferin at *p*H 7.6. The spectra are normalized to 1 at peak emission.

These results indicate that the complex of enzyme with intermediate is important in determining the nature of the transition complex in light emission. The alteration induced by temperature, *p*H, urea, and substrate analogs can modify the nature of the enzyme-bound emitter.

Reaction of dehydroluciferin with coenzyme A. Certain aspects of luciferase action can be studied more readily by considering a third catalytic reaction, namely, the formation of dehydroluciferyl-adenylate (L-AMP) from dehydroluciferin (L) and ATP (reaction 6).

The equilibrium constant for the activation step at *p*H 7.1 is 2.5×10^5, and the dissociation constant, *K*, as defined by the equation below was 5×10^{-10}.

$$K = \frac{(E)_{free}\,(L\text{-}AMP)_{free}}{(E \cdot L\text{-}AMP)}$$

This tight binding of dehydroluciferyl-adenylic acid to the enzyme explains why it is such a potent inhibitor of luciferase for light emission (*20*).

As it does with other synthetases, inorganic pyrophosphate readily reacts with the bound acyl-adenylate to form free L and ATP (reaction 6). Reduced coenzyme A also reacts with enzyme bound L-AMP to form dehydroluciferyl coenzyme A and adenylic acid (*45*) (reaction 7).

It has been demonstrated that L-coenzyme A in the presence of the enzyme, will react with AMP to form E·L-AMP. If ^{14}C-labeled AMP and ^{32}PP are added to such a reaction mixture, both labels can be recovered in the ATP. The specificity of the coenzyme A reaction is indicated by the fact that dephospho coenzyme A as well as other derivatives of coenzyme A are completely inactive. Other sulfhydryl compounds, such as cysteine and glutathione, will not react with L-AMP when it is bound to the enzyme although both compounds react rapidly with free L-AMP to form the corresponding dehydroluciferyl derivatives.

The reaction of coenzyme A with the enzyme-bound L-AMP occurs much more readily when ATP is present. This observation, as well as others discussed later, indicate that ATP induces a change in the luciferase structure in

such a way that the bound L-AMP becomes more reactive to certain molecules (Fig. 2). The increased reactivity of bound acyl-adenylates in the presence of ATP or other modifier molecules has been observed with other acyl-adenylate synthetases.

Reaction of E·L-AMP with water: hydrolase. When dehydroluciferin reacts with ATP in the presence of luciferase, an equilibrium is rapidly reached in which inorganic pyrophosphate is formed as one of the products along with enzyme bound L-AMP (reaction 6). If inorganic pyrophosphatase is added to the reaction mixture in order to hydrolyze the pyrophosphate, one observes a continuous breakdown of ATP into AMP and inorganic phosphate (*46*). This could only occur if the enzyme-bound L-AMP were hydrolyzed to form L, AMP, and free enzyme. Numerous experiments indicate that ATP does not favor the dissociation and slow hydrolysis of L-AMP. Rather, in the presence of ATP there is a rapid enzymatic hydrolysis of L-AMP (reaction 12) (*20*, *47*).

$$E \cdot L\text{-}AMP + H_2O \xrightarrow{ATP} E + L + AMP \quad (12)$$

Therefore ATP induces a change in the luciferase so that bound L-AMP reacts much more readily with water or coenzyme A. The formation of L-AMP on the enzyme apparently leads to a conformational change in the E·L-AMP complex that allows the steric accommodation of a second ATP (Fig. 2). This interaction of ATP with E·L-AMP brings out hydrolase activity. A similar hydrolase activity induced by tRNA has been observed by Norris and Berg for the isoleucyl-tRNA synthetase (*15*). This enzyme will activate both isoleucine and valine but does not catalyze the transfer of valine to tRNA. If isoleucine tRNA is added to enzyme-bound valyl-AMP, there is a rapid hydrolysis, with the formation of free valine and AMP. It is possible that hydrolase properties of the synthetases may be important in regulating or restricting the reactivity of bound acyl-adenylates.

Reaction of Fatty Acyl Coenzyme A Synthetase

After Berg (*5*) demonstrated that synthetic acetyl-AMP could serve as a substrate for the acetyl coenzyme A synthetase, numerous efforts were made to demonstrate the enzymatic formation of the intermediate from ATP and acetate. Webster and Campagnari were the first to actually demonstrate the formation of acetyl-^{14}C–adenylate from acetate-^{14}C, ATP, $MgCl_2$, and the synthetase (*17*). The fact that large quantities of enzyme were required suggested that the product was tightly bound to the enzyme. The equilibrium appeared to be in favor of ATP and free acetate. However, the removal of pyrophosphate by pyrophosphatase action did not increase the amount of acetyl-AMP formed. That the acetyl-^{14}C–adenylate was bound to the enzyme was suggested by the fact that there was a constant ratio between radioactivity and enzyme in the various fractions collected after the reaction mixture was chromatographed on a Sephadex column. In addition, the enzyme-associated radioactivity was converted to acetyl-^{14}C–coenzyme A by incubation with coenzyme A. If one assumes that one acetyl-adenylate combined with one active site in the enzyme, Webster was able to determine the maximum molar concentration of acetyl-adenylate needed to saturate the enzyme (*16*). From these data he calculated a molecular weight that agreed with the estimates determined by sedimentation characteristics (83,000 to 85,000).

Webster (*48*) has crystallized this enzyme and shown that the molecular weight of the catalytic unit is 35,000. This interesting finding of a difference in the molecular weight was due to the aggregation of enzyme molecules in the earlier preparation. Apparently in the aggregated preparation some of the catalytic sites were unreactive, as revealed by the higher specific activity of the crystalline preparation. It is evident that caution should be exercised when working with partially purified preparations. Reports of multiple synthetases for a specific substrate might reflect a similar aggregation-deaggregation phenomenon. Furthermore, it should be emphasized that dissociation of such an aggregate in vivo, if it occurs, could increase enzymatic activity by increasing the number of active sites rather than increasing the activity by some allosteric transition.

The enzyme purified by Webster showed an absolute requirement for Mg^{++} for the formation of acetyl-adenylate from ATP and acetic acid. The formation of acetyl-adenylate from acetyl coenzyme A and AMP, the reverse of reaction 9, did not depend on Mg^{++} ion. Furthermore, the yield of acetyl-adenylate under equilibrium conditions was not altered, indicating further that Mg^{++} ion was not essential for its binding. Similar results have been obtained by Campagnari and Webster using a highly purified acetyl-coenzyme A synthetase from bovine heart mitochondria (*39*). Adenylic acid inhibits the enzyme while adenosine diphosphate and Pi are without effect. Neither guanosine triphosphate or cytosine triphosphate would substitute for ATP, and glutathione would not substitute for coenzyme A.

Others studying synthetases which activate fatty acids of intermediate chain length have also concluded that specific fatty acyl-adenylates are enzyme-bound intermediates, formed from ATP and the corresponding acid (*31*, *49*).

Reaction of Aminoacyl-tRNA Synthetases

Properties of the reaction. It now seems certain that the initial steps in the synthesis of proteins involve the activation of the 20 amino acids by at least 20 specific synthetases to form bound aminoacyl-adenylates (reaction 10) (*5*, *50*). This activated amino acid is then transferred to a specific tRNA (sRNA) to form the aminoacyl-tRNA (reaction 11). It has been shown that these specific aminoacyl-tRNA's are recognized by a triplet base sequence in the messenger RNA (*51*). The activated amino acid is then incorporated into a polypeptide.

In addition to the role the aminoacyl-tRNA synthetases play in protein synthesis, they are important in the regulation of RNA synthesis, enzyme repression, and intergenic suppression (*52*). We have not attempted to review all aspects of these possible functions of the synthetases.

The aminoacyl-tRNA synthetases have been assayed by three major methods. One involves the amino acid–dependent ^{32}PP exchange (reaction 10), a second involves the formation of the hydroxamic acid derivatives of the amino acid, while the third method depends upon the transfer of amino acid to a specific tRNA (reaction 11).

After the earlier observations of Holley (*53*), Hoagland *et al.* (*54*), and Ogata and Nohara (*55*) there were many demonstrations of specific low-molecular-weight RNA (tRNA) fractions which would accept amino acids from specific synthetases (*50*). McCully and Cantoni (*56*) suggested a model for the structure of tRNA in which the

polynucleotide chain is folded back on itself like a hairpin. The unpaired nucleotides which must be present at the fold (the anticodon) were suggested as being available for specific hydrogen bonding to the appropriate triplet code on the messenger RNA, thus positioning the attached amino acid for polypeptide formation (*57*). The specificity of the tRNA in interacting with a specific aminoacyl-adenylate synthetase was presumed to be due to the secondary and tertiary structure of the tRNA.

It now seems certain that there are at least two important sites in each tRNA, each interacting specifically with the synthetase. One of these must be unique for each tRNA, the other common to all. The common site is the terminal adenosine which accepts the activated amino acid at the 3′-carbon of the ribose moiety. The unique site must involve the remaining structure of the polynucleotide.

Although little is known about the specific sites of interaction between the aminoacyl-tRNA synthetase and tRNA, a number of studies with either modified tRNA or various polynucleotides as inhibitors suggest that the anticodon is of importance in the recognition and the specificity of interaction (*58*). Other data indicate the importance of the entire structure of the RNA for specific interaction with enzyme-bound aminoacyl-adenylates (*59*).

A good example of the specificity of the transfer reaction has been shown by Norris and Berg using pure isoleucyl-tRNA synthetase from *Escherichia coli* (*15, 60*). This enzyme is unusual in that it is capable of forming both isoleucyl-adenylate and, to a lesser extent valyl-adenylate. However, when tRNA specific for isoleucine is added, only the isoleucyl-adenylate is transferred, while the valyl-adenylate is rapidly hydrolyzed. The addition of tRNA specific for accepting valine does not lead to the formation of valyl-tRNA. Thus even if the synthetase makes an error in recognizing and activating the wrong amino acid the specific tRNA is incapable of recognizing and reacting with the abnormal enzyme acyl-adenylate complex.

This same rigorous specificity has been observed in the reverse reaction. Herve and Chapeville (*61*) demonstrated this by preparing cysteinyl-tRNA and then reducing with borohydride to make alanyl-tRNA. Using the latter compound as a substrate and either cysteinyl-tRNA synthetase or alanyl-tRNA synthetase and AMP they were unable to demonstrate the formation of alanyl-adenylate.

Examining the same system as Norris and Berg, Loftfield and Eigner (*62*) have shown that the presence of tRNA specific for isoleucine suppresses valine activation and accelerates the rate of activation of isoleucine. Yeast tRNA, on the other hand, showed no stimulation, while oxidized *Escherichia coli* tRNA remained a potent activator for formation of isoleucine hydroxamate. These results suggest that tRNA specific for a given amino acid and its homologous synthetase induces a change in the enzyme resulting in increased specificity.

In some cases it is possible to alter the overall specificity of the reaction. Arca *et al.* (*63*) and Calvori *et al.* (*64*) have decribed the effect of temperature on the isoleucyl-tRNA synthesis in *Bacillus stearothermophilus*. At moderate temperature (50°C), both isoleucine and valine were activated by the enzyme but as reported by Norris and Berg (*15*) for the *Echerichia coli* enzyme, only isoleucine was esterified to the tRNA. However, as the temperature was increased to 80°C, the incorporation of isoleucine gradually diminished, while that of valine increased. Furthermore, it appeared that the tRNA for isoleucine was accepting valine since excess isoleucine decreased such incorporation. These data indicate a conformational change either in the enzyme or in the tRNA as the temperature is raised, resulting in an altered specificity. It is important to point out the analogy between the effect of temperature on this system and on luciferase where higher temperatures result in the emission of a red light instead of the normal yellow-green.

Because of the high degree of specificity of a given tRNA for its homologous enzyme, cross-reactions with heterologous systems would not be expected. A number of investigators have shown a varying degree of interchangeability of the synthetase and tRNA's in different organisms (*5, 50*). Only a few cases in which an absolute specificity exists have been found. These heterologous reactions have been partially explained by data indicating the presence of more than one tRNA specific for a given amino acid from a single species (*50*). It has been suggested that the anticodon of these tRNA's may be distinct (*65*). Chromatographic heterogeneity of tRNA preparations must not be used as the sole criteria for functionally different molecular forms, as shown by the experiments of Schleich and Goldstein (*66*). For example, they were able to convert, by treatment with urea and heat, one form of highly purified tRNA into another form with distinct chromatographic properties. In addition to the observed heterogeneity of the tRNA, there are also reports that the same organism has more than one synthetase capable of activating a single amino acid (*14, 50, 67*). From these observations, it has been suggested that the RNA charged by homologous enzymes is not the same as that charged by heterologous enzymes.

It is not possible to discuss here all the data concerned with the possible significance and mechanism of action of the anticodon structure in recognizing the specific aminoacyl-adenylate-synthetase complex. There are several recent reviews that present the extensive details (*50, 68*). The essential feature that unpaired bases in the tRNA are possibly important in recognizing specific amino acids derives primarily from our knowledge and thinking about the genetic code. As suggested by Crick, the tRNA must contain a complementary sequence for a code word in messenger RNA (*69*). Numerous workers have presented evidence which suggests that the base sequence on tRNA (anticodon) is also important for recognizing the amino acid adenylate bound to the enzyme. Dunnell has made a specific proposal concerning the possible mechanism of amino acid interaction with the anticodon (*70*).

These proposals do not consider the importance of the enzyme in restricting the reactivity of the bound aminoacyl-adenylate. In this sense the synthetase is not just a catalyst but is important in the specific recognition process. When the acyl-adenylate is formed on the enzyme we suggest that the tRNA must recognize a specifically induced region of the enzyme–aminoacyl-adenylate complex (Fig. 5). Furthermore, it seems likely that the terminal adenylate of the tRNA competes with the adenylate site of the acyl-adenylate and brings the accepting hydroxyl group in close proximity to the activated carboxyl group. The rapid interaction of AMP with aminoacyl-tRNA to form aminoacyl-adenylate and the latter interaction with PP to form ATP suggest a common or close binding site for the adenylate moieties of ATP, aminoacyl-adenylate, and aminoacyl-tRNA.

Reaction of hydroxylamine and pyrophosphate with enzyme aminoacyl-ade-

nylates. As indicated earlier, Loftfield and Eigner have demonstrated that homologous tRNA (*Escherichia coli*) markedly increased the rate of formation of isoleucyl hydroxamate while an equivalent amount of heterologous tRNA (yeast) had no effect. Isoleucyl-acceptor tRNA which had been oxidized to a point where it had less than 0.3 percent of its original ability to accept isoleucine still stimulated the hydroxamate formation. Thus the evidence indicates that the specific tRNA even without the amino acid (adenylate) acceptor capacity can change the enzyme–aminoacyl-adenylate complex so that it will react more readily with hydroxylamine. Loftfield and Eigner concluded that the tRNA caused a change in the conformation of the enzyme to bring about this effect.

Hele (*71*) and Hele and Barth (*72*) have also suggested that tRNA induced an allosteric conformational change in the aminoacyl-adenylate synthetase which results in an increased rate of incorporation of PP into ATP. Thus the evidence suggests an increased reactivity to both pyrophosphate and hydroxylamine when tRNA is added to the synthetase.

Reaction of nucleoside polyphosphates with enzyme aminoacyl-adenylates. Zamecnik *et al.* and Randerath *et al.* have demonstrated that the *E. coli* lysyl-tRNA synthetase will catalyze the formation of diadenosine tetraphosphate (APPPPA or AP_4A) (*73*). Lysine is essential for the synthesis, and tRNA is stimulatory. They propose that the terminal phosphate of ATP reacts with the enzyme-bound lysyl-adenylate much in the same manner as pyrophosphate reverses the initial activation step to form ATP (Fig. 5). In support of this mechanism they could demonstrate the formation of diadenosine triphosphate (AP_3A) when ADP was added to the reaction mixture. Furthermore "hybrid" nucleotides could be formed if, in addition to ATP, GTP or dGTP (deoxygua-

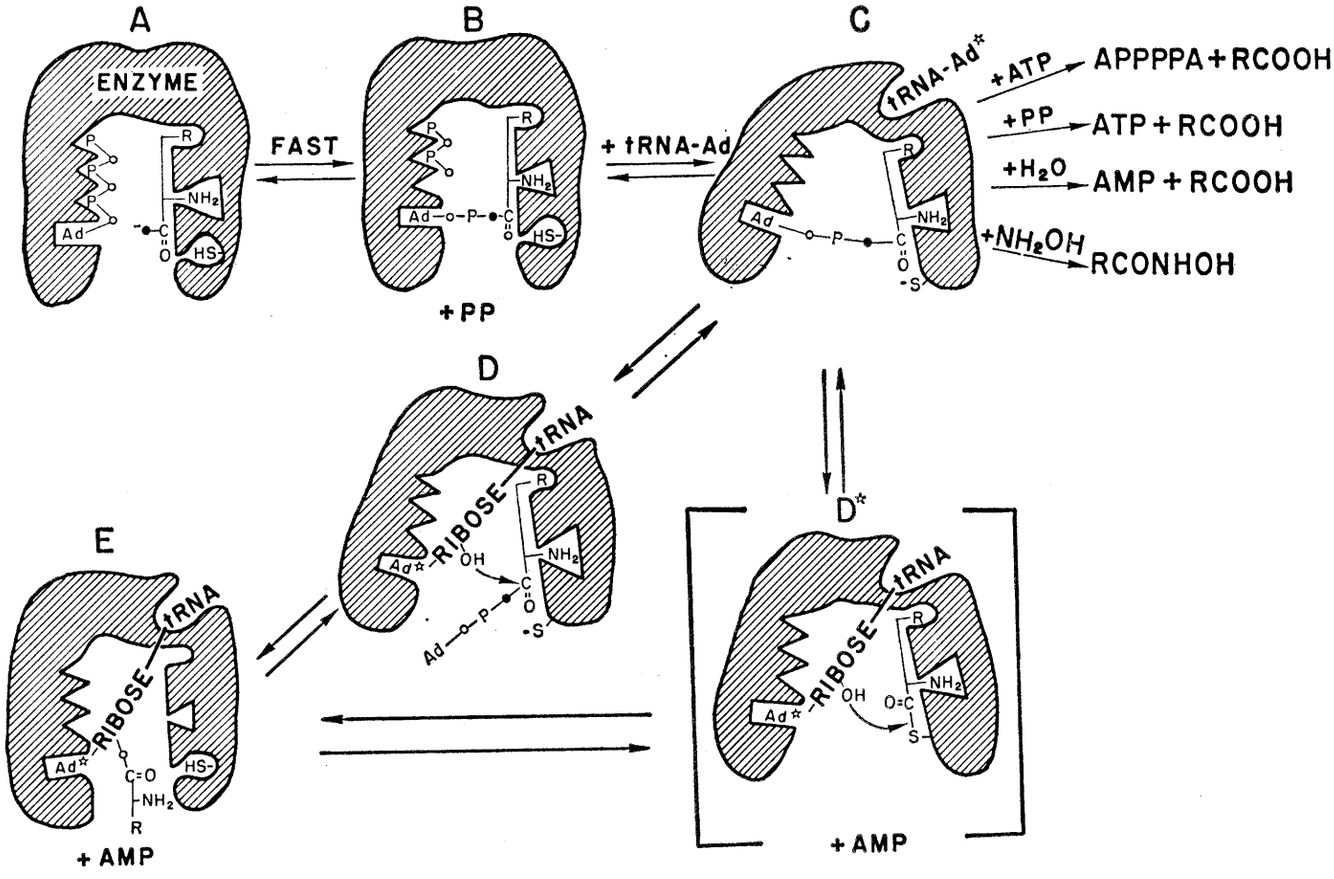

Fig. 5. Scheme of aminoacyl-adenylate synthetase activity. There are at least three important binding sites on the synthetases which catalyze the activation and transfer of amino acids. One of these sites is specifically concerned with the binding of ATP in which it is known that the adenine and pyrophosphate structures are essential for reactivity. The second site is specific for the amino acid in which the amino and carboxyl groups are common to all of the amino acids except proline. The initial reaction is the formation of aminoacyl-adenylate and pyrophosphate. A specific tRNA binds to the enzyme and recognizes the aminoacyl-adenylate–enzyme complex. Both the anticodon regions and the secondary structure of the tRNA appear to be important in the site recognition. In addition, the terminal adenosine which accepts the activated amino acid presumably binds at or near the adenosine site that was formerly occupied by the ATP. The binding of the tRNA to the enzyme alters the reactivity of the aminoacyl-adenylate toward a number of compounds. In the cases studied, the intermediate readily reacts with ATP to form diadenosine tetraphosphate (APPPPA), with PP to form ATP, or with water to form AMP and free amino acid, and with hydroxylamine to form hydroxamate. In many cases studied, tRNA is essential or very stimulatory for these reactions.

Several authors have suggested that sulfhydryl groups may be important in the formation of a thioester intermediate during the transfer reaction (*71, 81*). Boyko and Fraser, studying rat liver glycyl-tRNA synthetase, postulated a mechanism whereby presumably the aminoacyl-adenylate reacts with an -SH group on the enzyme to form a bound thioester, with the concomitant release of AMP. The specific tRNA then interacts with the thioester forming the aminoacyl-tRNA. The experimental evidence does not support this suggestion since no AMP-ATP exchange is observed in the absence of tRNA. This is also true for other synthetases, that is, coenzyme A is required for AMP-ATP exchange with luciferase (*45*) and acetyl-coenzyme A synthetase (*8*). It is, however, possible that the tRNA interacts with the enzyme–aminoacyl-adenylate complex inducing a thioester formation as indicated above.

nosine triphosphate) is added to the reaction mixture. An important observation is that dATP (deoxyadenosine triphosphate) can substitute for ATP in the aminoacylation reaction. The suggestion is made that these dinucleoside tetraphosphates may be an important reservoir of nucleoside triphosphate for the synthesis of other macromolecules such as RNA and DNA. It would be of interest to determine whether water (hydrolase activity) competes at all with ATP in the splitting of lysyl-adenylate. That the terminal pyrophosphate structure is needed suggests, in effect, that it competes for the pyrophosphate recognition site. Adenylic acid apparently is inactive, since no diadenosine diphosphate is formed.

Biotin and Lipoic Acid Synthetases

The enzymes which activate biotin and lipoic acid have been studied extensively by several groups (*74*). These enzymes characteristically catalyze the formation of the acyl-adenylates as the initial step in the incorporation of biotin and lipoic acid into protein. However, the transfer step is unusual in that the acyl-adenylate interacts directly with a particular amino group on the apoenzyme, resulting in the formation of the holoenzyme. Coon *et al.* have isolated a biotin synthetase from pig liver and from bacteria, which is not the same as the enzyme isolated by Lynen *et al.* (see *74*) since it will not catalyze the formation of propionyl coenzyme A holocarboxylase. Instead, this synthetase activates biotin to form biotinyl-adenylate, and then causes its transfer to coenzyme A to form biotinyl coenzyme A, a reaction similar to those described for luciferase.

All of the above enzymes have a specific requirement for ATP and Mg^{++}, and are capable of catalyzing either an ATP-dependent P-P exchange or hydroxamate formation. They, therefore, have similar properties to all previously described synthetases.

Conformational Changes of Luciferase During Catalysis

Conformational changes of an enzyme accompanying the binding of substrates are an important aspect of enzyme catalysis. We have cited examples of altered reactivity of acyl-adenylate synthetases in the presence of various substrates or analogs.

Table 1. Optical rotation parameters of luciferase in the presence and absence of substrates*.

	b_o	% helix	225 mμ		193 mμ	
			A	% helix	A	% helix
Native	−235*	37	−795	38	762	42
Native + substrates	− 90*	14	−352	15	272	28

* A value of −630 was used for 100-percent helix. The data presented are based on dispersion measurements in the region 600 to 300 mμ.

Generally, the Michaelis-Menten constant (K_m) for both ATP and acyl compounds is higher for the overall reaction from activation through the transfer than for the formation of enzyme-bound acyl-adenylate as measured by the PP-ATP exchange (*5, 50*). In addition, the presence of an acceptor molecule influences the activation reaction, possibly by altering the conformation of the enzyme.

Such studies provide indirect evidence of possible conformational changes of the enzyme during catalysis. The synthetases constitute a class of proteins with an unusually high affinity for the acyl-adenylate intermediate. It might be suggested that this tight binding of the acyl-adenylate to the enzyme is the result of an induced folding of a portion of protein around the substrate, thus excluding the water molecules or other nucleophiles from direct contact with the bound substrate. One sensitive method for detecting such a "folding" is to measure the rates of tritium-hydrogen exchange of the enzyme in the presence and absence of substrates (*75*). In small molecules, such as amino acids, the hydrogens attached to oxygen, nitrogen, or sulfur exchange readily with the hydrogens of water. In macromolecules such as proteins, the rates of exchange of hydrogens are often much slower. These rates appear to be a function of the local environment of the hydrogen within the macromolecules, of the *p*H and of the temperature. Under specified conditions, the total number of exchanging hydrogens can be resolved into classes of definite size and with a specific rate of exchange. These properties are characteristic of the specific macromolecule, and they reflect the time the enzyme spends in an exchanging configuration.

The rates of hydrogen-tritium exchange have been measured for luciferase in the presence and absence of ATP and L (*76*). The results (Fig. 6) demonstrate a large difference in the exchange rates in the presence of substrate. This reflects a large change in the structure of the protein. If these nonexchanging hydrogens are amide hydrogens, an estimated 39 percent of the protein becomes inaccessible to water in the presence of substrates.

Another method of determining changes in macromolecules is the measurement of the optical rotatory dispersion spectra (*77*). Through the appropriate analyses of such spectra it is possible, in some cases, to obtain an estimate of how much of the protein molecule has the α-helical structure. The data obtained for luciferase in the presence and absence of substrates are given in Table 1. If these analyses of the spectra are a true reflection of the helical content, the figures show that in the native enzyme there is 37 to 40 percent helix (*78*). The addition of substrates results in a marked decrease of the apparent helical content to about 14 percent. This is in agreement with the exchange data and shows that large structural changes of the enzyme occur during catalysis. Although a detailed interpretation is not possible, these experiments represent the only direct evidence for such changes in synthetases.

Additional indirect data suggesting large changes in conformation come from observations on the thermal inactivation of luciferase in the presence and absence of substrate. The results presented in Table 2 demonstrate that the free energy of activation ($\Delta F‡$) is increased in the presence of the substrate; that is, there is substrate protection against thermal inactivation. The changes in heat of activation ($\Delta H‡$) and in entropy of activation ($\Delta S‡$) are remarkably different. Even though the $\Delta F‡$ in the presence of substrate is

Table 2. Thermal inactivation of luciferase and pyrophosphatase.

Agent	$\Delta F‡$ (42°C) (cal/mole)	$\Delta H‡$ (cal/mole)	$\Delta S‡$ (e.u.)
Luciferase	20,000	103,000	262
Luciferase + L + ATP	22,700	46,300	75
Pyrophosphatase	18,000	57,400	125
Pyrophosphatase + PP	20,000	124,000	316

increased, it is evident that the $\Delta S\ddagger$ is greatly reduced (262 to 75). Although a quantitative interpretation is not possible, it is evident that we are dealing with a different form of the enzyme in the presence of substrate and that the path of thermal inactivation has been significantly altered. We believe that these changes in the thermodynamic properties of thermal inactivation are due to the large conformational alterations in the enzyme induced by the substrates. The changes are quite different for the thermal inactivation of inorganic pyrophosphatase in the presence and absence of substrate, which could imply a different type of conformational change when the substrate combines with this enzyme.

The large conformational changes which occur in luciferase in the presence of substrate are of interest when we consider the role of sulfhydryl groups in catalysis. We have shown previously that the presence of substrates prevents the reaction of two sulfhydryl groups in luciferase from reacting with sulfhydryl reagents. The two substrate-protected sulfhydryl groups in luciferase have been shown to be essential for enzymatic activity. Examination of the two sulfhydryl groups covered by substrate led to the isolation of a single decapeptide containing one sulfhydryl group where two different peptides might have been expected (*24*). Since two subunits had previously been demonstrated, the results suggest that mutual cooperation of two cysteinyl residues from apparently identical monomers in forming at least part of the active site of luciferase. However, in view of the large conformational changes that occur during catalysis it is possible that the two substrate-protected sulfhydryl groups are "buried" in the nonaqueous structure of the enzyme and are therefore unreactive. If this explanation is correct, these sulfhydryls may not be part of the active site. Even so, in view of the similarities in amino acid composition of the synthetases, it would still be of interest to compare the amino acid sequence in the vicinity of the critical sulfhydryl residues in these enzymes.

In all of these studies that indicate large conformational changes, we have not observed the dissociation of luciferase into its two subunits. Only in the presence of high concentrations of urea or guanidine hydrochloride does luciferase dissociate into two equal subunits.

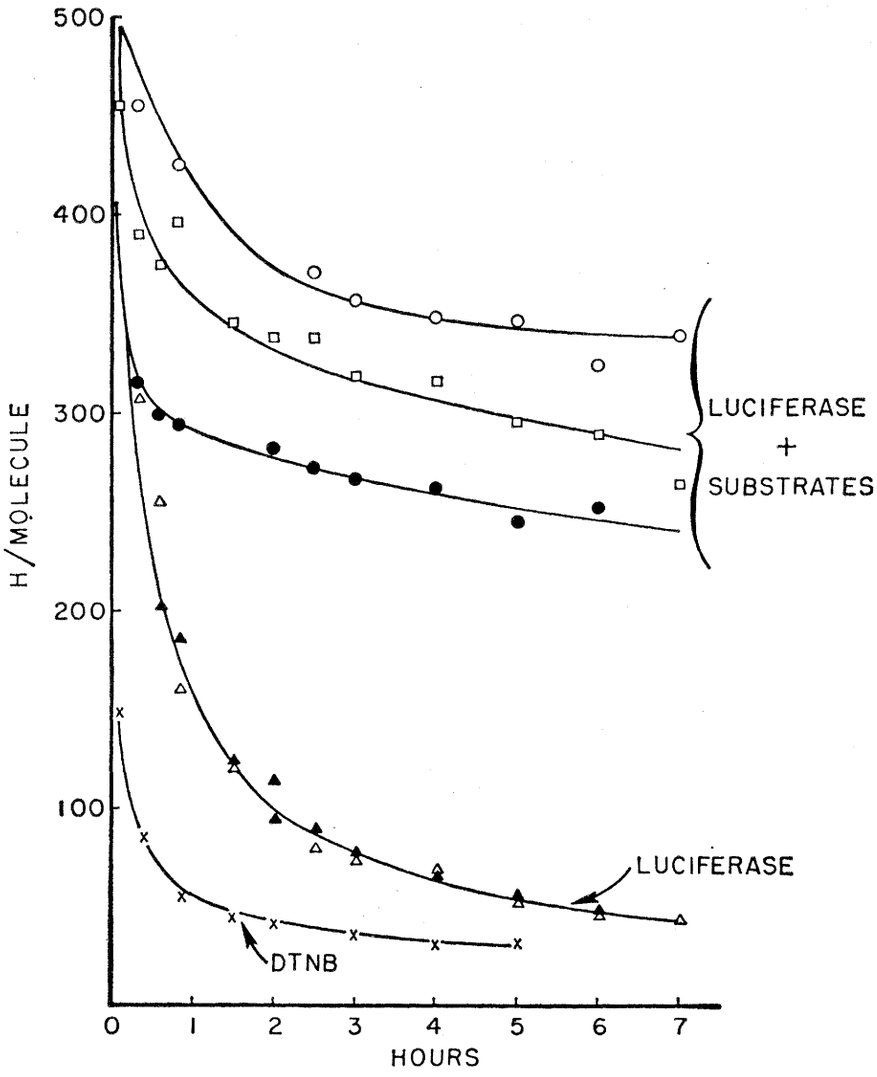

Fig. 6. Tritium-hydrogen exchange of luciferase in the presence and absence of substrates. The luciferase was tritiated for 12 hours at 4°C, *p*H 7.0. Tritium exchange was followed as a function of time. The number of exchangeable hydrogens per molecule of luciferase are plotted on the ordinate. △ and ▲, Duplicate experiments with native luciferase; ○ and □, duplicate experiments with native luciferase in the presence of dehydroluciferin (L) and ATP; ●, experiment performed with a luciferase of lower specific activity; ×, a luciferase made catalytically inactive by titrating the SH groups with DTNB.

Homosterism

The results of numerous investigations on luciferase and other acyl-adenylate synthetases indicate that large conformational changes occur in the protein when the specific substrates combine at the active site. In addition, the reactivity of the enzyme-bound acyl-adenylates is greatly altered when a second substrate or normal ligand is bound at the catalytic center.

For example, enzyme-bound dehydroluciferyl-adenylate does not readily react with coenzyme A unless ATP is added. In the absence of coenzyme A, ATP alters the luciferase structure in such a way that water can readily react with L-AMP, thus hydrolyzing it to free L and AMP. The net effect is that luciferase becomes a hydrolyase for the continued breakdown of ATP into AMP and inorganic pyrophosphate.

We propose the term homosterism to describe those cases in which a normal substrate or structurally similar compound combines at the catalytic site of the enzyme and leads to a modification of the reaction of the bound intermediate. This is distinguished from allosterism where the modifier molecule

combines at a site other than the catalytic center (*79*). It is also different from the induced fit concept of Koshland (*80*) in that we are proposing that it is the enzyme bound intermediate whose reactivity is altered by its interaction with a second ligand. It is evident from the data discussed that the initial reaction of the enzyme with the substrate molecules may involve an induced fit mechanism as judged by the large conformational changes that take place. However, a homosteric transition occurs when another substrate molecule interacts with the enzyme-bound acyl-adenylate.

In addition to the hydrolase properties of luciferase, which are induced by ATP, we suggest that the change in the color of light emitted, when the ATP isomer mentioned earlier is used instead of ATP, is another example of a homosteric effect of a ligand. Numerous studies on the aminoacyl-adenylate synthetases indicate that tRNA induces a homosteric transition in these enzymes, thus altering the reactivity of the bound aminoacyl-adenylate. For example, the studies of Norris and Berg indicate that isoleucine tRNA alters the corresponding synthetase in a way that bound valyl-adenylate is rapidly hydrolyzed.

In addition, Loftfield and Eigner (*62*) demonstrated that tRNA increased the reactivity of the aminoacyl-adenylate to hydroxylamine. They suggest that the tRNA induces an allosteric conformational change; however, we propose that this is an example of a homosteric effect, since it is the combination of a normal substrate molecule at the catalytic center. Hele and associates have observed similar effects of tRNA on the pyrophosphate exchange reaction.

Zamecnik, Randerath, and associates have shown that tRNA will greatly stimulate the reaction of ATP with enzyme-bound lysyl-adenylate to form diadenosine tetraphosphate. The arginyl- and glutamyl-tRNA synthetases show an absolute requirement for a homosteric transition in that tRNA is essential for the formation of the aminoacyl-adenylate.

In all cases cited above, the results suggest a homosteric rather than an allosteric effect. We suggest that the homosteric transition brought about by natural substrates acting at the catalytic site is important in regulating the specificity and reactivity of enzyme-bound acyl-adenylates.

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Creative Tensions in the Research and Development Climate

Technical achievement of scientists and engineers was high under conditions that seemed antithetical.

Donald C. Pelz

What kinds of climate in research and development organizations are conducive to technical accomplishment? What is the optimum degree of freedom versus coordination? of pure research versus practical development? of isolation versus communication? of specialization versus diversification?

To find some answers, my colleagues and I studied 1300 scientists and engineers in 11 research and development laboratories. Since the answers in different kinds of settings might vary, we included five industrial laboratories, five government laboratories, and seven departments in a major university. Their objectives ranged from basic research to product development.

Among the findings appeared a number of apparent inconsistencies. The optimum climate was not necessarily some compromise between extremes. Rather, achievement often flourished in the presence of factors that seemed antithetical.

Some examples are given below and summarized in Table 1 (*1*). As we pondered these findings, it seemed possible to fit many of them under two broad headings. On the one hand, technical men were effective when faced with some demand from the environment—when their associates held divergent viewpoints or the laboratory climate required disruption of established patterns. These might be called conditions of challenge.

On the other hand, technical men also performed well when they had some protection from environmental demands. Factors such as freedom, influence, or specialization offer the scientist stability and continuity in his work —conditions of security.

It seemed reasonable to say that the scientists and engineers of our study were more effective when they experienced a "creative tension" between sources of stability or security on the one hand and sources of disruption or challenge on the other. The term was suggested by T. S. Kuhn in a paper entitled "The essential tension: tradition and innovation in scientific research" (*2*).

Necessity is said to be the mother of invention, but our data suggest that invention (technical achievement) has more than one parent. Necessity might better be called the father—since necessity is one form of challenge, a masculine component. The role of mother is, rather, some source of security. When both are present, the creative tension between them can generate scientific achievement.

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Methods

The findings were not obtained by polling scientists concerning what climate they preferred. Rather, we obtained measures of each man's scientific performance, including his scientific or technical contribution to his field of knowledge in the past 5 years, as judged by panels of his colleagues; his overall usefulness to the organization, through either research or administration, also as judged by his colleagues; the number of professional papers he had published in the past 5 years (or, in the case of an engineer, the number of his patents or patent applications); and the number of his unpublished reports in the same period.

The performance measures were modified in several ways. Since distributions of papers, patents, and reports were skewed, a logarithmic transformation was applied to normalize them. Systematic variations with level of education, length of working experience, time in the organization, and type of institution were removed by adding constants so as to equalize the means. Each scientist, that is, was scored relative to others with similar background.

Characteristics of the climate were obtained on a carefully tested questionnaire. The two sets of data (on performance and on climate) were analyzed to find those conditions under which scientists actually performed at a higher or lower level.

Since optimum conditions might differ in different settings, all analyses were replicated within five subcategories: Ph.D.'s in research-oriented laboratories; Ph.D.'s in development-oriented laboratories; non-Ph.D.'s in research-oriented and in development-oriented laboratories (for convenience the latter have been called "engineers"); and non-Ph.D.'s in laboratories where 40 percent or more of the staff members held a doctoral degree (because of the limited influence and promotional opportunity of these non-Ph.D.'s we have called them "assistant scientists").