It is particularly worth noting that the spectra of unperturbed NO–Hb, NO–Mb, and NO–cytochrome *c* belong to the C type, in which the strong interaction between Fe and the base molecule (histidine in the hemoproteins) was found operating through π as well as σ orbitals as is manifested by the g_y value and the super hyperfine splitting, respectively. If we realize that NO–Hb molecule would perhaps be a nearest available model system of oxyhemoglobin, it seems obvious that the characteristic electronic structure of imidazole group has to be realistically taken into account, along with that of the sixth ligand, in any theoretical description of the mechanism for reversible oxygen binding of hemoglobin and myoglobin.

Furthermore, the present finding that the unpaired electron in NO–hemoproteins occupies the out-of-plane σ^* orbital, and that it is strongly delocalized out of the NO group, places severe restrictions on any theoretical model which attempts to explain the bonding of O_2 and NO to hemoglobin and myoglobin.

References

Ballhausen, C. J. (1962), Introduction to Ligand Field Theory, New York, N. Y., McGraw-Hill.

Falk, J. E. (1964), Porphyrins and Metalloporphyrins, New York, N. Y., Elsevier.

Gibson, J. F., and Ingram, D. J. E. (1957), *Nature 180*, 29.

Griffith, J. F. (1957), *Nature 180*, 31.

Griffith, J. F. (1964), *Biopolymer Symp. 1*, 35.

Helcké, G. A., Ingram, D. J. E., and Slade, E. F. (1968), *Proc. Roy. Soc. (London) B169*, 275.

Kneübuhl, F. K. (1960), *J. Chem. Phys. 33*, 1074.

Kon, H. (1968), *J. Biol. Chem. 243*, 4350.

Kon, H. (1969), *Biochem. Biophys. Res. Commun. 35*, 423.

McConnell, H. M., and Robertson, R. E. (1957), *J. Phys. Chem. 61*, 1018.

McGarvey, B. R. (1967), *J. Phys. Chem. 71*, 51.

McNeil, D. A. C., Raynor, J. B., and Symons, M. C. R. (1965), *J. Chem. Soc.*, 410.

Morell, D. B., and Stewart, M. (1956), *Australian J. Exptl. Biol. 34*, 211.

Sands, R. H. (1955), *Phys. Rev. 99*, 1222.

Shiga, T., Hwang, K. J., and Tyuma, I. (1969), *Biochemistry 8*, 378.

Watson, R. E. (1959), Technical Report, MIT Solid-State and Molecular Theory Group, No. 12, June 15.

Zerner, M., Gouterman, M., and Kobayashi, H. (1966), *Theoret. Chim. Acta (Berlin) 6*, 363.

Requirement of Different Sulfhydryl Groups in the Activation and Transfer Reactions of Isoleucyl Transfer Ribonucleic Acid Synthetase*

Tuan Kuo and M. DeLuca†

ABSTRACT: Purified isoleucyl transfer ribonucleic acid synthetase isolated from *Escherichia coli* catalyzed two reactions: (I) the conversion of isoleucine and adenosine triphosphate into isoleucyl adenylate–enzyme complex (the activation reaction); (II) the transfer of isoleucine from the complex to transfer ribonucleic acid. The role of the sulfhydryl groups in the enzyme was studied using *p*-mercuribenzoate and 5,5′-dithiobis(2-nitrobenzoic acid) as inhibitors. The native enzyme contained 10–11 titratable sulfhydryl groups. One or two of these sulfhydryls reacted very rapidly with 5,5′-dithiobis(2-nitrobenzoic acid). The loss of these rapidly reacting sulfhydryl groups resulted in complete inability to catalyze reaction I. Titration of the isolated isoleucyl adenylate–enzyme complex with *p*-mercuribenzoate resulted in a loss of ability to transfer the isoleucine to transfer ribonucleic acid (reaction II), while the ability to catalyze reaction I was essentially unaffected. The results demonstrate that different sulfhydryl groups in the enzyme are involved in reactions I and II.

One of the common properties of the aminoacyl-tRNA synthetases is their requirement for intact enzyme sulfhydryl groups (McElroy *et al.*, 1967; Novelli, 1967). The only synthetase not inhibited by sulfhydryl reagents is the *Escherichia coli* lysyl-tRNA synthetase (Stern *et al.*, 1966). In the case of the tryptophanyl-tRNA synthetase from beef pancreas, it has been shown that the presence of ATP–Mg and tryptophan prevents four of eight sulfhydryls from reacting with DTNB[1] (DeLuca and McElroy, 1966),

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[1] Abbreviations used in this paper are: NEM, *N*-ethylmaleimide; PMB, *p*-mercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Ile-AMP–E, the complex formed with isoleucyl-tRNA synthetase and isoleucyl adenylate; tRNA-AMP, the terminal AMP of tRNA; BSA, bovine serum albumin.

and the four protected sulfhydryls are essential for enzymatic activity. Similarly George and Meister (1967) showed that valyl-tRNA synthetase from *E. coli* contains 16 sulfhydryl groups that react with PMB. The presence of substrates prevents the reaction of eight of these sulfhydryls and protects the enzymatic activity. Finally, another enzyme which also forms an enzyme-bound acyl adenylate (reaction I), firefly luciferase, contains two sulfhydryls that are protected by the substrates (DeLuca *et al.*, 1964; Lee and McElroy, 1969) and again these protected sulfhydryls are essential for activity. Thus, it is possible that most synthetases contain one or more sulfhydryls which are required for catalysis.

The isoleucyl-tRNA synthetase from *E. coli* has been reported (Baldwin and Berg, 1966) to contain 15 half-cystine residues, eight of which are reactive with DTNB. In the presence of isoleucine and ATP–Mg, approximately four of these sulfhydryls do not react with DTNB.

The present studies extend those previously reported on the requirement of sulfhydryl groups for the activity of *E. coli* isoleucyl-tRNA synthetase. The results indicate that the enzyme contains two distinguishable sulfhydryl groups that participate in the over-all reaction, the incorporation of isoleucine into tRNA.

Materials and Methods

Substrates. [^{32}P]PP_i was purchased from Tracerlab; [^{14}C]-ATP was from Schwarz BioResearch and [^{14}C]isoleucine was obtained from New England Nuclear Corp. Crude *E. coli* tRNA was purchased from General Biochemicals. *E. coli* strain B was obtained from Grain Processing Corp., Muscatine, Iowa.

Isoleucyl-tRNA synthetase was purified from *E. coli* B by a modification of the procedure described by Baldwin and Berg (1966). The final specific activity of the enzyme was comparable with that previously reported. Disc electrophoresis (Ornstein and Davis, 1964) indicated that over 95% of the enzyme–protein migrated as single component. When [^{14}C]-Ile-AMP–E complex was isolated from Sephadex G-75 column (Norris and Berg, 1964), the molar ratio of [^{14}C]isoleucine to enzyme was found to be 1.0.

Enzyme Assays. Two assays for enzymatic activity were used; the activation step (I) was assayed by measuring the incorporation of [^{32}P]PP_i into ATP in the presence of isoleucine. One unit of activity is defined as the incorporation of 1 μmole of [^{32}P]PP_i into ATP in 15 min at 37° (Baldwin and Berg, 1966) using the assay conditions described by Mehler and Stern (1966).

The ability of the enzyme to catalyze the incorporation of [^{14}C]isoleucine into tRNA was assayed as described by Mehler and Stern (1966). Assay of the enzyme by both methods showed that the rates of the two reactions were constant with time of incubation and directly proportional to protein concentration with the ranges that were used.

Sulfhydryl Determinations. Sulfhydryl groups were determined with PMB and DTNB. The reaction of the enzyme with DTNB was followed by measuring absorbancy changes at 412 mμ, and the SH content was calculated using a molar extinction coefficient of 13,600 (Ellman, 1959). The titration of the SH groups with PMB was conducted by the method of Boyer (1954). In a typical experiment, 0.25–0.35 mg of enzyme was diluted to 1.0 ml with 0.1 M Tris buffer (pH 7.1), and PMB (approximately 1×10^{-3} M) was added in 5-μl increments to both the blank and sample cells. The absorbancy at 250 mμ was followed until no further change occurred, at which time an additional 5 μl of PMB was added. The first five to six SH's reacted almost instantaneously, while the remaining four or five SH's required up to 45 min for complete reaction. In some experiments SH determinations were performed in the presence of substrates; here, the blank cell contained substrates in addition to buffer. The SH content of an identical sample of enzyme lacking substrates was always determined simultaneously.

The PMB and DTNB stock solutions were standardized by titration of BSA; the results obtained with the two reagents agreed within 3%, and also agreed with theory.

Protein concentration was measured by the method of Lowry *et al.* (1951). Crystalline BSA was used as a standard. In 0.1 M phosphate buffer (pH 7.9), an enzyme solution with an optical density at 280 mμ of 1.0 contains approximately 0.7 mg of protein/ml.

All of the calculations are based on a molecular weight of 112,000 for the enzyme (Baldwin and Berg, 1966).

Formation of Ile-AMP–E Complex. The Ile-AMP–E complex was prepared as described by Norris and Berg (1964), except that 2-mercaptoethanol and glutathione were omitted from both the reaction mixture and eluting buffer. The specific activity of the [^{14}C]isoleucine in the reaction mixture was 17 μCi/μmole. In some experiments, [^{14}C]ATP of approximately the same activity was used to prepare doubly labeled complex. Routinely 0.8–1.6 mg of purified enzyme was used in the incubation and the Ile-AMP–E complex was isolated by fractionation on a Sephadex G-75 column. Labeled fractions were counted in a liquid scintillation fluid (Bray, 1960) in a Nuclear-Chicago liquid scintillation spectrometer at an efficiency of 70%.

Reaction of Isolated Ile-AMP–E Complex with PMB. A small DEAE-cellulose column (0.6 × 3 cm), described by Grosjean *et al.* (1968), was used for following the breakdown of Ile-AMP–E by PMB. The reaction mixture (0.3 ml) contained 0.35 mumole of [^{14}C]Ile-AMP–E in 0.05 M succinate buffer (pH 6.0) and 60 mμmoles of PMB, and was maintained at 0° for the specified lengths of time. In other experiments, when limiting amounts of PMB were used, the incubation was also at 0° but for 1 hr. The mixture was then placed on the DEAE-cellulose column and eluted with T-1, T-2, and T-3 buffers as previously described (Grosjean *et al.*, 1968). With this procedure, free isoleucine or isoleucyl adenylate and excess PMB were not absorbed on the column and are found in the T-1 buffer. The [^{14}C]Ile-AMP–E complex was eluted with T-2 buffer. Fractions were collected and the radioactivity was determined on aliquots as described above. The total recovery of radioactivity from the column was 90%.

Reaction of Isolated [^{14}C]Ile-AMP–E Complex with tRNA. Transfer of the amino acid from the isolated complex to tRNA was also determined with the DEAE-cellulose column method described above. The reaction mixture (0.4 ml) in 0.05 M succinate (pH 6.0), contained 0.1 mμmole of ^{14}C complex, 20 μmoles of $MgCl_2$, and 1 mg of crude tRNA. After 45 min at 0°, the reaction mixture was placed on the column, eluted successively with T-1, T-2, and T-3 buffers, and the fractions were assayed for ^{14}C. Aminoacyl-tRNA elutes with T-3 buffer. With this assay control samples transferred 50–60% of the total [^{14}C]isoleucine from the complex to the tRNA. In

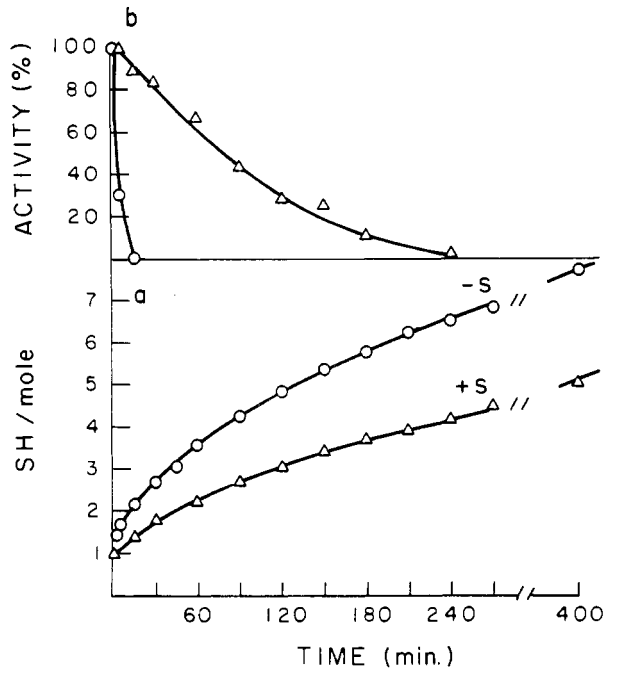

FIGURE 1: The reaction of DTNB with isoleucyl-tRNA synthetase. Enzymatic activity was measured by the [^{32}P]PP_i–ATP-exchange reaction. The cuvets contained 0.312 mg of enzyme in 1 ml of 0.1 M phosphate buffer (pH 7.9). The enzyme–substrate sample had 2×10^{-3} M ATP, 4×10^{-3} M $MgCl_2$, and 8×10^{-4} M isoleucine. At zero time approximately 0.3 μmole of DTNB was added to all cuvets and the optical density was followed at 412 mμ as a function of time (a). At the times indicated, 0.01 ml of sample was removed for assay (b). (O—O) Native enzyme; (Δ—Δ) enzyme in the presence of substrates.

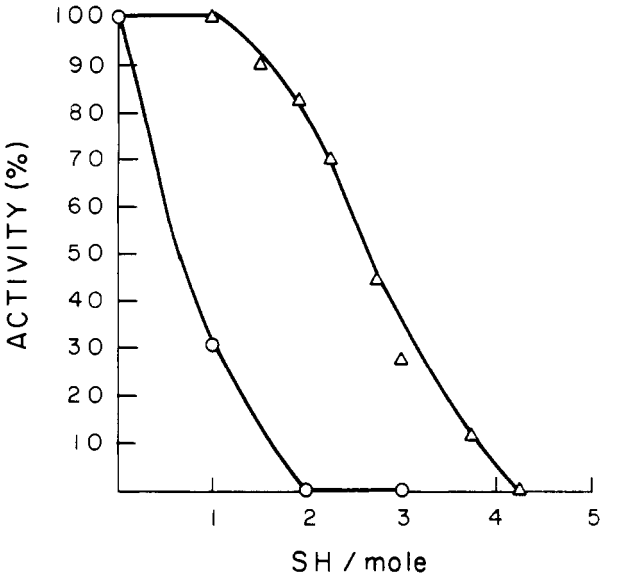

FIGURE 2: [^{32}P]PP_i–ATP-exchange activity of isoleucyl-tRNA synthetase as a function of the number of sulfhydryls reacted with DTNB. The data are taken from the experiments shown in Figure 1. (O—O) Native enzyme; (Δ—Δ) enzyme in the presence of substrates.

contrast to the report of Norris and Berg (1964), we found that $MgCl_2$ stimulated the transfer approximately fourfold.

Results

The rate of the reaction of isoleucyl-tRNA synthetase with DTNB at room temperature is shown in Figure 1a. After 6.5 hr about eight SH groups reacted per mole of native enzyme. The presence of ATP–Mg and isoleucine prevented the reaction of approximately 2.5 SH's during this time. The loss of activity in the native enzyme was very rapid (Figure 1b). In the presence of substrates, activity was lost at a much slower rate, requiring 4-hr reaction with DTNB for complete inhibition. Figure 2 shows the loss of [^{32}P]PP_i–ATP-exchange activity as a function of the number of SH reacted with DTNB. By the time 2 SH's/mole have reacted in the native enzyme, there was no detectable activity by the [^{32}P]PP_i–ATP-exchange assay. In the presence of substrates, 4 SH's/mole must react with DTNB for complete inhibition. After 6.5-hr reaction with DTNB both the native enzyme and the enzyme plus substrates were put into 5 M guanidine (pH 7.9) in the presence of DTNB; both samples were shown to have 10 reactive sulfhydryls/mole. Therefore no sulfhydryls have been irreversibly lost during the incubation with substrates.

Analysis of the kinetics of the reaction of enzyme with DTNB indicate that at the earliest measurable point, about 2 min, 1.5 SH's/mole have reacted in the native enzyme. These rapidly reacting sulfhydryls will be designated the "fast SH" for following discussion. In the presence of substrates only 1.0 SH/mole reacts instantaneously. The remaining SH groups react in a typical first-order manner with a $t_{1/2}$ of 90 min. The presence of substrates did not affect the rate of these reactions.

In another experiment, tRNA was added to the enzyme prior to DTNB. Approximately 2 SH's/mole were protected from reacting in the presence of RNA (Table I) but the RNA did not protect the fast SH which is essential for the [^{32}P]PP_i–ATP exchange (Figure 2). Since large amounts of crude tRNA were used in this experiment, we do not know whether the protection resulted solely from the presence of isoleucine tRNA. Enzyme assays performed in the presence of varying concentrations of AMP (Figure 3) demonstrated that the transfer reaction is inhibited to a greater extent by a given concentration of AMP than the [^{32}P]PP_i–ATP-exchange reaction. Analysis of the kinetics of AMP inhibition demonstrated that it is competitive with respect to tRNA. If the AMP is inhibiting the charging of tRNA by driving the reaction back toward Ile-AMP–E, then a larger concentration of enzyme should result in greater apparent inhibition of transfer activity. This was not observed. AMP (5×10^{-8} M) resulted in the same amount of inhibition over a tenfold range of enzyme concentration.

When the SH groups were determined in the presence of this concentration of AMP, it was found the AMP protected 1–1.3 SH's/mole. These data are shown in Table I. AMP did not protect the fast SH, and therefore did not protect the [^{32}P]PP_i–ATP-exchange activity. This is consistent with the inhibition data (Figure 3), *i.e.*, there is an AMP binding site which is inhibitory for the transfer reaction and to a lesser extent the exchange reaction. The substrates do not protect any SH groups from reaction with PMB. As can be seen from Figure 4, the extinction of the mercaptide, as reflected in the final absorbancy, is lower for the enzyme–substrate sample although the equivalence point is essentially the same. The average number of sulfhydryl groups reacting with PMB is

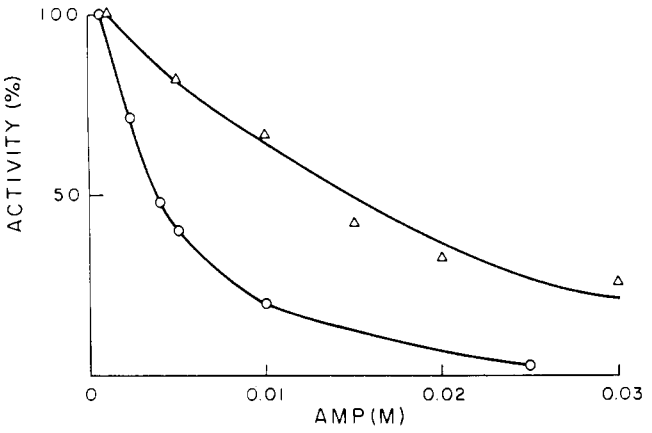

FIGURE 3: The inhibition of isoleucyl-tRNA synthetase by AMP. The enzyme was assayed for [^{32}P]PP_i–ATP-exchange activity (Δ—Δ) and transfer activity (O—O) as described in Methods. AMP was included in the assay tubes at the final concentration indicated. The activity is expressed as per cent of a control which contained no AMP.

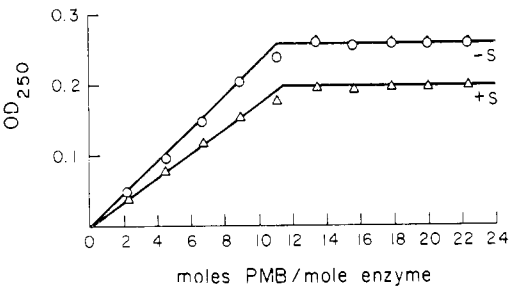

FIGURE 4: PMB titration of the sulfhydryl groups of isoleucyl-tRNA synthetase in the presence and absence of substrates. The cuvets contained 0.35 mg of enzyme in 1.2 ml of 0.1 M Tris buffer (pH 7.1). The lower curve, marked +S, contained 1.67×10^{-4} M ATP, 3.2×10^{-4} M $MgCl_2$, and 2.3×10^{-4} M isoleucine. The PMB was 1.39×10^{-3} M and was added in 5-μl aliquots. The optical density at 250 mμ was followed until each aliquot of PMB had completely reacted.

10.6 ± 1/mole. This value was obtained from eight separate titrations. In several experiments it was found the PMB reacted with approximately two more sulfhydryls per mole than DTNB. This value of 10.6 is in agreement with a maximum number of 11 SH's/mole reacting with NEM as previously reported (Baldwin and Berg, 1966). Figure 5 shows the loss of [^{32}P]PP_i–ATP-exchange activity as a function of the number of sulfhydryls reacted with PMB. In the native enzyme, 73% of the activity is lost after three sulfhydryls have reacted. There is no further loss of activity as more sulfhydryls react (see Discussion). The substrates do show a decided protective effect in the early stages of the titration. When 4.3 SH groups have reacted with PMB, the enzyme is still 90% active. Therefore we conclude that the first sulfhydryls reacting in the native enzyme must be different from those which react in the presence of substrates. This result is similar to those obtained with DTNB (Figure 2).

TABLE 1: Sulfhydryl Content of the Isoleucyl-tRNA Synthetase in the Presence of Various Substrates.[a]

Native	Isoleucine + ATP–Mg	+RNA	+AMP	Difference
7.3	4.8			2.5
7.75	5.1			2.65
7.0		4.5		2.5
8.5			7.4	1.1

[a] Sulfhydryls were determined using DTNB as described in Methods. Where indicated isoleucine was present at a final concentration of 8×10^{-4} M, ATP was 2×10^{-3} M; $MgCl_2$, 4×10^{-3} M; crude tRNA, 4 mg; and $MgCl_2$, 2×10^{-2} M; AMP was 5×10^{-3} M. In all experiments the value reported for the native enzyme was determined at the same time as the values obtained with the various substrates. Units given in SH per mole.

The effect of PMB on the formation of the Ile-AMP–E is shown in Table II. Incubation of excess PMB with the enzyme either in the presence or absence of substrates markedly inhibits the formation of the complex. Other experiments were conducted to determine the effect of PMB on the stability of the isolated [^{14}C]Ile-AMP–E complex. The isolated complex was incubated at 0° in the presence and absence of excess PMB for various times from 15 min to 3 hr. The reaction mixtures were then analyzed by DEAE-cellulose chromatography as described above. Figure 6 shows the elution pattern of a control and a sample of [^{14}C]Ile-AMP–E which was incubated for 3 hr with excess PMB. The total ^{14}C counts applied to the column were recovered in two peaks: the first one is a breakdown product and the second peak is the [^{14}C]Ile-AMP–E complex. In the control experiment all of the counts were recovered as active complex as measured by the ability to transfer [^{14}C]isoleucine to tRNA. The time course for the breakdown of the complex by PMB is shown in Figure 7; the half-life of the complex is 75 min at 0° and pH 6.0 in the presence of excess PMB.

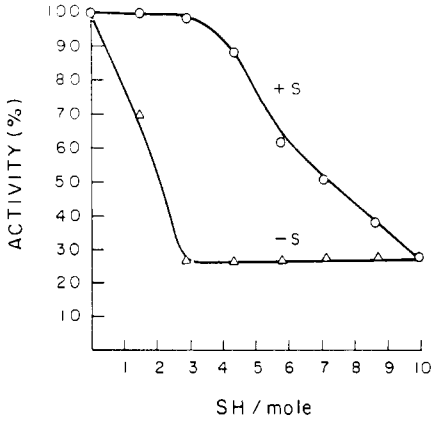

FIGURE 5: Effect of PMB on the activity of isoleucyl-tRNA synthetase, in the presence (O—O) and absence of substrates (Δ—Δ). Experimental conditions are the same as in Figure 4. After each addition of PMB, absorbancy at 250 mμ was followed until there was no further increase. An aliquot was removed from both samples and assayed for enzymatic activity by [^{32}P]PP_i–ATP exchange.

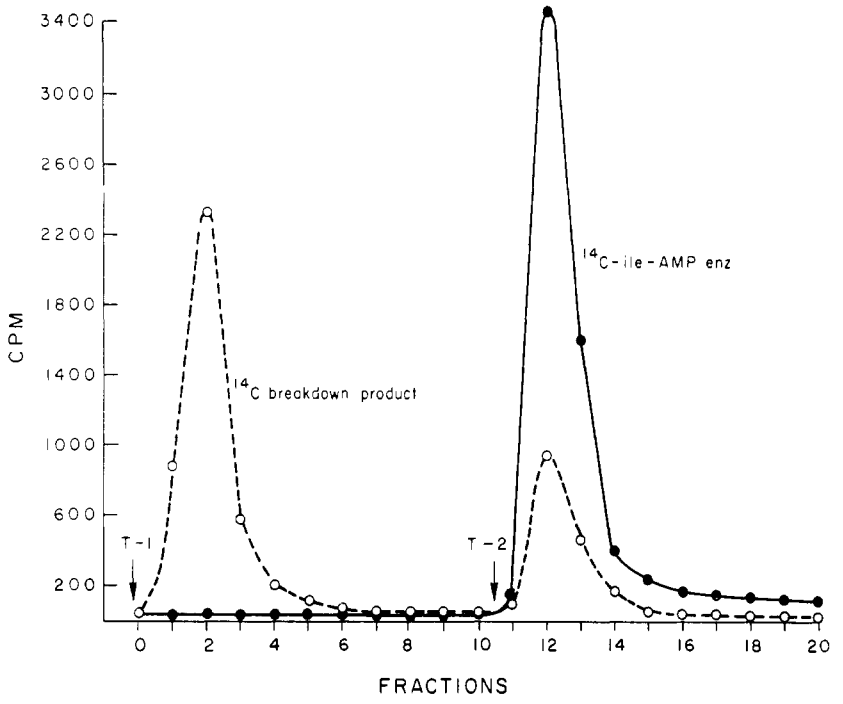

FIGURE 6: The breakdown of [^{14}C]Ile-AMP–E by PMB. The isolated [^{14}C]Ile-AMP–E complex was incubated in the presence (-O- -O-) or absence (●—●) of excess PMB for 3 hr in succinate buffer (pH 6.0). The reaction mixture was then fractionated by stepwise elution on DEAE-cellulose column described in the Methods.

After 3-hr reaction with PMB all of the complex is broken down; however it is not clear whether this release of counts is due to the dissociation of [^{14}C]Ile-AMP from the enzyme or to the hydrolysis of the complex to free [^{14}C]isoleucine and AMP. To distinguish between these two possibilities the doubly labeled [^{14}C]Ile-[^{14}C]AMP–E complex was isolated and incubated with PMB. The breakdown product was chromatographed on DEAE-cellulose with added unlabeled AMP as a marker. Only one peak of radioactivity was detected, and it was separated from the AMP. When this radioactive peak was hydrolyzed (pH 9.0, 37°) for 30 min and rechromatographed, two peaks of radioactivity were eluted from the column. One corresponded to [^{14}C]isoleucine, and the other to [^{14}C]AMP. These experiments demonstrated that PMB released isoleucyl adenylate from the enzyme without hydrolysis.

When PMB was added to the isolated [^{14}C]Ile-AMP–E complex, 1 equiv at a time, no breakdown of the complex was observed during a 30-min incubation. As shown in Figure 8, when 7 SH's/mole had reacted, the ability of the complex to transfer isoleucine to tRNA was essentially completely inhibited. However, and most important, the enzyme was still able to catalyze isoleucine activation as measured by the exchange assay, and retained almost full activity for this reaction. This experiment demonstrated that the site responsible for the amino acid activation and the site required for the formation of isoleucyl-tRNA are different.

TABLE II: PMB Effect on the Formation of Ile-AMP–E Complex.[a]

Conditions	cpm as Isolated [^{14}C]Ile-AMP–E Complex	% Complex Formation
Enzyme + substrates (control)	12,340	100
Enzyme + substrates + PMB, 1 hr	1,700	14
Enzyme + PMB, 1 hr then substrates	744	6

[a] Three series of experiments were performed. In the first experiment (the control), 1.5 mμmoles of isoleucyl-tRNA synthetase was incubated with 2 μmoles of Mg^{2+}, 1 μmole of ATP, 0.15 μmole of [^{14}C]isoleucine (specific activity 17 μCi/μmole), 0.4 μmole of potassium phosphate buffer (pH 7.5). After 1 hr at room temperature the [^{14}C]Ile-AMP–E complex was isolated as described in Methods. The second experiment was identical with the first one, except that the incubation mixture had 64 mμmoles of PMB in addition to the substrates. In the third experiment, the enzyme was incubated with PMB alone for 1 hr at room temperature then the substrates were added just 5 min before isolation of the complex.

Discussion

The observation that PMB can completely inhibit the transfer of isoleucine to tRNA without significantly affecting the [^{32}P]PP_i–ATP exchange (Figure 8) is evidence for at least two distinguishable classes of sulfhydryl groups in the enzyme, each of which is essential for a different catalytic activity. One of these SH groups must be required for the transfer activity, the other one or possible two SH groups is necessary for the activation of isoleucine and the [^{32}P]PP_i–ATP-exchange activity (Figure 2). The ability of 5×10^{-3} M AMP to significantly inhibit the transfer reaction (Figure 3) and also to protect approximately one SH from reacting with DTNB supports the hypothesis that this SH group is involved in binding the

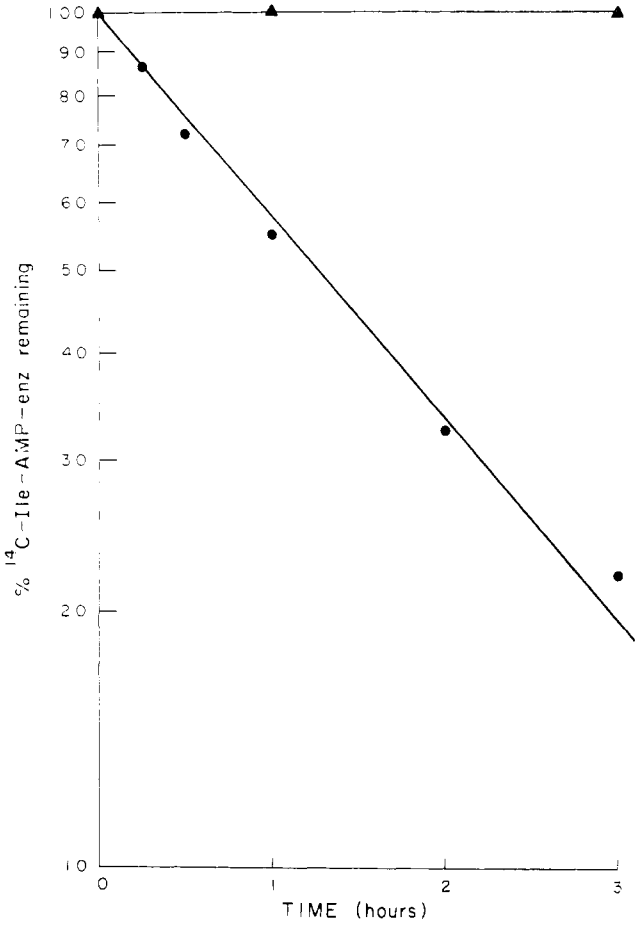

FIGURE 7: Time course of the breakdown of [^{14}C]Ile-AMP-E complex by PMB. Isolated complex was incubated at 0° in the presence (●) and absence (Δ) of excess PMB for different lengths of time. The breakdown of the complex by PMB was followed by using a DEAE-cellulose column as described in Methods.

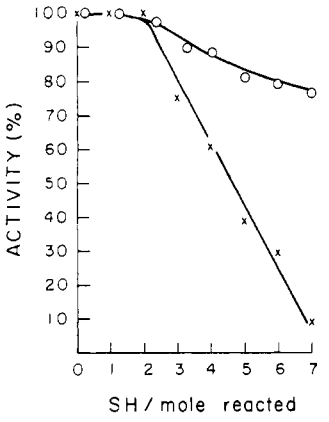

FIGURE 8: Titration of [^{14}C]Ile-AMP-E with limiting amounts of PMB. Isolated [^{14}C]Ile-AMP-E complex was divided into eight aliquots. To each 0.24 mμmole of complex increasing amounts of PMB were added such that the molar ratio of PMB to complex was from 1 to 7. The control had no PMB. After 1 hr at 0° the aliquots of each sample were assayed for [^{32}P]PP_i–ATP exchange (○—○) and transfer of amino acid to tRNA (x—x) as described in Methods. Each point represents the average of four experiments (○) or two experiments (x). There was little or no breakdown of the complex during the experiments. Thus after 1 hr with 7 equiv of PMB, only 7% of the complex had broken down.

terminal AMP of tRNA. This interpretation is consistent with the observation that neither AMP nor tRNA protect the one or two very reactive SH groups required for the exchange activity (Figure 2). On the basis of these experiments we propose separate binding sites for the terminal AMP of tRNA and the AMP portion of the isoleucyl adenylate.

Cassio (1968) has reported a similar effect on a partially purified methionyl-tRNA synthetase from *E. coli*. She found that in the presence of methioninyl adenylate, a structural analog of the normal substrate, PMB completely inhibits the transfer reaction while the activation reaction was uninhibited.

In contrast to other synthetases (DeLuca and McElroy, 1966; DeLuca *et al.*, 1964; George and Meister, 1967), the presence of ATP–Mg and isoleucine does not prevent the reaction of sulfhydryls with PMB (Figure 4). The significance of the residual 27% [^{32}P]PP_i–ATP-exchange activity after all the sulfhydryls have reacted with PMB (Figure 5) is not known. A possible explanation is that when the PMB–enzyme is diluted out for assay (1000 times) some of the PMB dissociates from the enzyme. Another possibility is that the sulfhydryls do not participate directly in catalysis but are required to maintain the catalytic site in an optimal configuration.

The data indicate that PMB displaces the isoleucyl adenylate from the enzyme rather than including hydrolysis of the bound acyl adenylate as was observed with firefly luciferase (DeLuca and McElroy, 1965). The simplest interpretation of these experiments is that the PMB is reacting with the sulfhydryls thus preventing the binding of isoleucyl adenylate. The possibility that the excess PMB effects a conformational change of the protein which results in dissociation of isoleucyl adenylate can not be excluded.

Other investigators have reported inhibition of the transfer of amino acids to tRNA by approximately 1×10^{-3} M PMB (Allende *et al.*, 1966; Hirsh, 1968). It is known that PMB reacts with tRNA at these concentrations (Stern *et al.*, 1966) so the interpretation of such inhibitions is not clear. In all of the experiments reported in this paper, the excess PMB was removed prior to adding tRNA for the transfer activity assay.

The reaction of the enzyme with DTNB (Figures 1 and 2) shows that the native enzyme contains 1–1.5 very reactive sulfhydryl groups and that these are the sulfhydryls required for exchange activity. In contrast to the results obtained with PMB, the presence of isoleucine and ATP–Mg prevents the reaction of DTNB with two to three SH groups.

The observation that $MgCl_2$ stimulates the transfer of amino acid to tRNA (see Methods) is not in agreement with previous reports (Norris and Berg, 1964). Grosjean *et al.* (1968) have recently found the isoleucyl-tRNA synthetase from *Bacillus stearothermophilus* does require Mg^{2+} for transfer. These authors also report some hydrolysis of the complex in the presence of tRNA. These results are in agreement with the experiments reported here. When the isolated complex was reacted with tRNA it was found that 50–60% of the [^{14}C]isoleucine was transferred, while the additional 40% of the complex was hydrolyzed.

The experiments reported here demonstrate that modification of different sulfhydryls in the isoleucyl-tRNA syn-

thetase results in the inhibition of different catalytic activities. Such experiments do not prove that the sulfhydryls participate directly in catalysis or in the binding of substrates. The experiments do give evidence for two distinct sites on the enzyme, one of which is required for the activation of the amino acid, and another for the transfer of amino acid to the tRNA.[2]

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References

Allende, C. C., Allende, J. E., Gatica, M., Celis, J., Mora, G., and Matamala, M. (1966), *J. Biol. Chem. 241*, 2245.
Baldwin, A. N., and Berg, P. (1966), *J. Biol. Chem. 241*, 831.
Boyer, P. D. (1954), *J. Am. Chem. Soc. 76*, 4331.
Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Cassio, D. (1968), *European J. Biochem. 4*, 222.
DeLuca, M., and McElroy, W. D. (1965), *Biochem. Biophys. Res. Commun. 18*, 836.
DeLuca, M., and McElroy, W. D. (1966), *Arch. Biochem. Biophys. 116*, 103.
DeLuca, M., Wirtz, G., and McElroy, W. D. (1964), *Biochemistry 3*, 935.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
George, H., and Meister, A. (1967), *Biochim. Biophys. Acta 132*, 165.
Grosjean, H., Charlier, J., and Vanhumbeeck, J. (1968), *Biochem. Biophys. Res. Commun. 32*, 935.
Hirsh, D. I. (1968), *J. Biol. Chem. 243*, 5731.
Iaccarino, M., and Berg, P. (1969), *J. Mol. Biol. 42*, 151.
Lee, R., and McElroy, W. D. (1969), *Biochemistry 8*, 130.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
McElroy, W. D., DeLuca, M., and Travis, J. R. (1967), *Science 157*, 150.
Mehler, A. H., and Stern, R. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y., Harper & Row, pp 414–415.
Norris, A. T., and Berg, P. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 330.
Novelli, G. D. (1967), *Ann. Rev. Biochem. 3*, 449.
Ornstein, L., and Davis, B. (1964), *Ann. N. Y. Acad. Sci. 121*, 321, 404.
Stern, R., DeLuca, M. Mehler, A. H., and McElroy, W. D. (1966), *Biochemistry 5*, 126.

[2] Since the submission of this paper, Iaccarino and Berg (1969) have presented data implicating the involvement of one sulfhydryl of the isoleucyl-tRNA synthetase in the formation of isoleucyl adenylate. They have also suggested that other sulfhydryls may be involved in binding of tRNA to the enzyme. Although the experiments differ from those presented in this paper the conclusions reached are in agreement with ours.