Glutamine Phosphoribosylpyrophosphate Amidotransferase. Catalytic and Conformational Heterogeneity of the Pigeon Liver Enzyme*

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ABSTRACT: Further studies are reported on the allosteric enzyme, glutamine phosphoribosylpyrophosphate amidotransferase, from pigeon liver. The enzyme demonstrated sigmoid kinetics with respect to the specific substrate 5-phosphoribosyl 1-pyrophosphate, the sigmoidicity being accentuated by the negative effector adenosine 5′-monophosphate. Examination of a wide variety of purified enzyme preparations revealed at least four different catalytic forms of the enzyme. One form of the enzyme was catalytically inactive, but was activated by the specific substrate 5-phosphoribosyl 1-pyrophosphate and Mg^{2+}. The inactive protein has been designated the state I form of the enzyme, and the activated enzyme the state II form.

The state I form of the protein was highly sensitive to iron chelation, but this sensitivity was greatly reduced by the activation to state II. The negative effector, adenosine 5′-monophosphate, protected the purine ribonucleotide sensitive state I enzyme from inhibition by the chelator, but was ineffective if the enzyme was insensitive to ribonucleotide inhibition. Another form of the enzyme is referred to as the state II′ form. This variety was catalytically active without preincubation with phosphoribosylpyrophosphate and Mg, relatively insensitive to chelators and was protected by adenosine 5′-monophosphate whether it was sensitive to it or not. Both of the catalytically active forms (II and II′) showed a cooperative effect on the addition of the other substrate, glutamine, with a progressive acceleration of the reaction velocity until a maximum was achieved. Under these conditions a fourth form of the enzyme existed, the state III form. This form was completely insensitive to iron chelation. The fluorescent hydrophobic probe, 2-*p*-toluidinylnaphthalene-6-sulfonate, supported the interpretation that substrates induce conformational changes in the enzyme. The conditions for these changes are different for state II′ and state I enzymes. The implications of these findings are discussed.

Glutamine phosphoribosylpyrophosphate amidotransferase (EC 2.4.2.14 ribosylamine 5-phosphate:pyrophosphate phosphoribosyltransferase) catalyzes the first committed step in purine biosynthesis, the β substitution of the amido group of glutamine on C-1 of phosphoribosylpyrophosphate (PP-ribose-P). PP-ribose-P is a specific substrate for the enzyme, but glutamine is relatively nonspecific since various other substrates will react to form their corresponding ribose 5′-phosphate derivatives. Evidence exists that certain of these other substrates, such as ammonium chloride and methanol, do not bind at the glutamine site (Hartman, 1963a). The enzyme partially purified from both pigeon liver (Caskey *et al.*, 1964) and from bacteria (Nierlich and Magasanik, 1965) satisfies the first two criteria for an allosteric enzyme defined by Koshland and Neet (1968), *viz.*, the enzyme (1) contains a site topologically distinct from the active site, (2) catalyzes a reaction at a branch point in a metabolic pathway, (3) demonstrates sigmoid kinetics, and (4) obeys the symmetry or concerted model. The studies outlined here indicate cooperative binding of the specific substrate PP-ribose-P, with classical sigmoid kinetics accentuated by the negative effector AMP. Sigmoid kinetics in the presence of AMP can also be deduced from data on the bacterial enzyme (Nierlich and Magasanik, 1965). The third criterion is thus satisfied.

Approaches to assessing the fourth criterion have been made. In previous work Rowe and Wyngaarden (1968) showed that the molecule of 200,000 mol wt dissociates into electrophoretically identical subunits of 50,000 mol wt in the presence of reducing agents. These subunits are highly unstable, but are enzymatically active in the assay system employed, and sensitive to inhibition by AMP and GMP. The molecular weight of the enzymatically active protein is unknown. Purine ribonucleotide sensitivity is a highly labile property which can be readily lost and occasionally regained during the course of purification. The enzyme contains iron, part of which is labile and appears to be a component of the active site (Hartman, 1963a; Rowe and Wyngaarden, 1968).

Examination of a large number of partially purified enzyme preparations disclosed interesting kinetic properties with a variable lag phase in the assay system employed and a coop-

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erative binding of the second substrate glutamine. Enzymes which demonstrated varying kinetic properties also revealed differences in their susceptibility to inhibition by iron chelators and in their responses to purine ribonucleotides.

Furthermore, the fluorescent hydrophobic probe, 2-*p*-toluidinylnaphthalene-6-sulfonate revealed that the conformational changes induced by addition of various substrates occur under different conditions with enzymes exhibiting different kinetic properties. This enabled us to outline a provisional model for the conformational states of the enzyme and to discuss possible implications of catalytic heterogeneity of this enzyme for biological control mechanisms.

Materials

The following chemicals were utilized in these studies: PP-ribose-P, AcPyDPN, AMP (P-L Biochemicals, Inc.); L-glutamine (Mann Research Laboratories); bovine liver glutamic acid dehydrogenase, crystalline in 50% glycerol, free of ammonium ions (Sigma Chemical Corp.); 1,10-*o*-phenanthroline (Fisher Scientific Co.); 2-*p*-toluidinylnaphthalene-6-sulfonate was synthetized according to McClure and Edelman (1966).

Methods

The *standard assay* for amidotransferase employed in this study involved measurement of the glutamate generated by coupling the reaction to the reduction of AcPyDPN in the presence of an excess of glutamic acid dehydrogenase (Wyngaarden and Ashton, 1959). The usual assay was performed at 25° in a volume of 1.0 ml containing 0.25 mM PP-ribose-P, 1.0 mM glutamine, 3.0 mM $MgCl_2$, 0.6 mM AcPyDPN, the enzyme, 50 mM Tris-Cl buffer (pH 8.0), and an excess of glutamic acid dehydrogenase (0.27 μmolar unit). The reduction of the pyridine nucleotide derivative was followed at 363 mμ in a Gilford recording spectrophotometer.

With this assay there is a lag phase of variable duration before the rate of the reaction becomes linear (Wyngaarden and Ashton, 1959). In order to determine whether this lag was a property of the coupled assay system two alternative assays were employed in selected studies.

Alternative Assay 1. A solution (12.0 ml) containing 0.25 mM PP-ribose-P, 3.0 mM $MgCl_2$, 1.0 mM glutamine, enzyme, and 50 mM Tris-Cl buffer (pH 8.0) was incubated at 37° and 1.0-ml samples were removed at fixed time intervals. The reaction was stopped by immersion of the sample in a boiling-water bath for 1 min and subsequent rapid cooling in an ice bath. To each of these samples was added AcPyDPN to a final concentration of 0.6 mM and 1.35 μmolar units of glutamic dehydrogenase. These were then incubated for 2 hr at 37°, the solution was clarified by centrifugation at 20,000*g* for 20 min, and the absorbance was measured at 363 mμ.

Alternative Assay 2. A radioisotope assay was run concurrently with the assay described above by the addition of 4.0 μCi of glutamine-^{14}C (Schwarz BioResearch, specific activity 16 mCi/mmole). When timed samples had been removed and the reaction was stopped, 10 μl of sample was applied to a 0.5-mm microcrystalline cellulose thin-layer plate. This was subjected to electrophoresis in a 0.1 M sodium acetate buffer (pH 6.0) for 30 min at 1000 V. Under these conditions glutamic acid moves toward the anode while glutamine moves slightly in a cathodal direction. The amino acids, localized with a ninhydrin spray, were removed, placed in vials containing 1,4-bis[2-(5-phenyloxazolyl)]benzene–2,5-diphenyloxazole–toluene scintillation fluid, and counted in a Packard liquid scintillation spectrometer with an efficiency of 80%. The rate of generation of glutamate from glutamine was calculated from the relative distribution of isotope between the two amino acids.

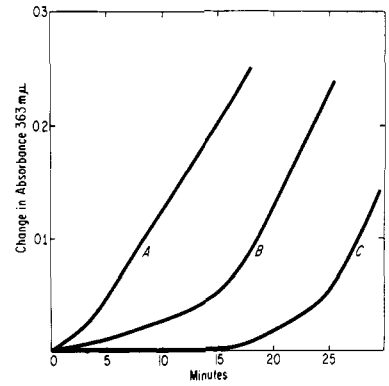

FIGURE 1: Kinetic curves of standard assays of three different enzyme preparations illustrating the marked variations in the time lag: (1) before measurable activity is obtained, and (2) before maximum velocity is attained.

A possible additional assay, based on release of pyrophosphate, was discarded because of the insensitivity introduced by the high background of carrier phosphate at low substrate levels and at short time intervals.

Enzyme used in all studies reported in this paper had been purified according to the technique of Rowe and Wyngaarden (1968), excluding only the final step of acrylamide chromatography. At this stage of purification there is little or no background reaction due to the nonspecific reduction of AcPyDPN in the standard assay, such as was observed with less highly purified preparations in earlier work (Wyngaarden and Ashton, 1959; Caskey *et al.*, 1964; Rowe and Wyngaarden, 1968).

Fluorometric studies were performed with an Aminco-Bowman spectrophotofluorometer.

Results

Lag Phase. Different enzyme preparations showed a highly variable lag phase before maximal velocity was achieved. This time lag ranged from a few minutes to 30 min or more. For any one preparation, at any one time, however, the lag phase was reproducible. Three activity patterns were seen (Figure 1). Curve A illustrates enzyme with considerable initial catalytic activity rising to maximal velocity within 5 min. Curve C illustrates the behavior of enzyme with no initial catalytic activity, followed by a steady rise after 15 min, finally attaining a maximum at 25 min. Curve B resembles an intermediate between A and C, with a low but definite initial velocity rising to a maximum at 15 min.

The lag phenomenon is not a property of the coupled assay system: (a) The time lag could be shortened considerably

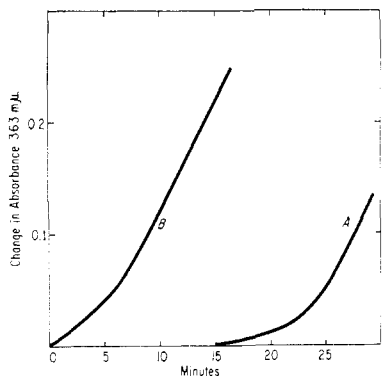

FIGURE 2: Kinetic curves representing the effect of a 20-min preincubation of state I enzyme with PP-ribose-P (2.5×10^{-4} M) and Mg^{2+} (3×10^{-3} M). State I enzyme (curve A) shows no demonstrable initial activity, a gradual rise from 15 min and a maximum at 25 min. After preincubation, demonstrable initial activity is seen with a maximum attained at 7 min (curve B).

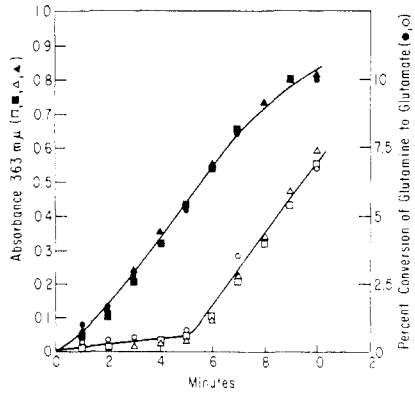

FIGURE 3: Kinetic curves demonstrating the consistency of results when different assays are employed. The open symbols represent results obtained with enzyme as prepared; the closed symbols with enzyme preincubated for 15 min as described in the legend of Figure 2. Data obtained with the standard assay are represented by squares, with alternative assay I by triangles, and with alternative assay II by circles. All assays were performed at 37°. This experiment was run to establish that the lag phase, whether long or short, was not an artifact of the kinetically coupled (standard) assay.

by incubation with the specific substrates PP-ribose-P and Mg^{2+} (Figure 2), but not with glutamine, glutamine, and Mg^{2+}, or by PP-ribose-P or Mg^{2+} alone. The final velocity attained with or without the preincubation was the same; this was important for the determination of half-maximal velocity values. (b) The lag phase was identical in the standard assay and in alternative assays I and II, both in the case of enzyme with a long lag phase and of enzyme preincubated with PP-ribose-P and Mg^{2+} (Figure 3). (c) The time lag prior to achievement of maximal velocity was constant for any one enzyme preparation regardless of the amount of amidotransferase activity present in the assay system (Figure 4). (d) The time lag was not modified in the standard assay by use of a 10-fold higher activity of glutamic dehydrogenase.

We observed also that the maximal velocity could never be attained initially regardless of the time of incubation, the concentration of substrates, or the assay system employed

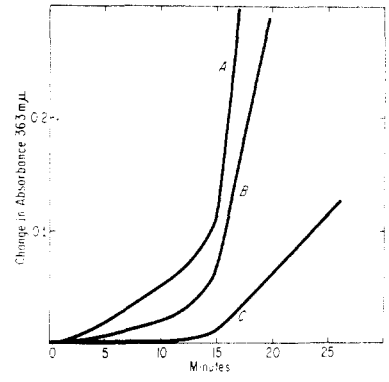

FIGURE 4: Kinetic curves demonstrating the independence of the lag phase from the amount of enzymatic activity added to the assay system as represented by the final velocities attained. Curves A and B represent ten- and fivefold, respectively, of the activity added to the assay represented by curve C.

(Figure 3). The results in these studies indicate that the enzyme was in a different form when the reaction was proceeding maximally.

With respect to catalytic activity, then, we have demonstrated *four* major forms of the enzyme (with the understanding, of course, that any one preparation may contain a mixture of these forms), *viz.*, (1) enzyme which initially was essentially inactive in the assay system and slowly gained activity in the presence of substrate, referred to as *state I* enzyme; (2) enzyme, initially inactive, which was incubated in the presence of PP-ribose-P and Mg^{2+} and subsequently demonstrated a definite initial velocity, as well as a shortened time lag before attaining maximal velocity–referred to as *state II* enzyme; (3) enzyme which demonstrated an immediate high rate of activity, albeit not maximal, in the assay system, referred to as *state II'* enzyme; (4) enzyme under conditions where all substrates were present and the reaction was proceeding maximally—referred to as *state III* enzyme.

Examination of Physical Properties of Different Forms of the Enzyme. Two questions arose in considering these descriptions of different forms of the enzyme as defined by kinetic properties. These questions related first to the reversibility of the changes between different forms of the enzyme and the factors responsible for these changes; and second, to other physical parameters that could be utilized to demonstrate possible conformational alterations without resorting to more formal physical studies demanding far greater quantities of highly purified enzyme than are currently available for this particularly labile molecule.

Form Interchange. If additional PP-ribose-P was added to the assay at the point where this substrate became *limiting*, the reaction resumed immediately, although there was the usual time lag before maximal velocity was attained. If state II' enzyme was used, the time lag was identical with that seen at the initial phase of the reaction (Figure 5, curve A). This indicated that the state III form of the enzyme had reverted to the state II' form. If state I or state II enzyme was used, the time lag corresponded with that seen with the state II form of the molecule (Figure 5, curve B).

If the reaction was allowed to proceed until PP-ribose-P was *exhausted* prior to the further addition of this substrate, a slightly different pattern emerged. With (state II' enzyme,

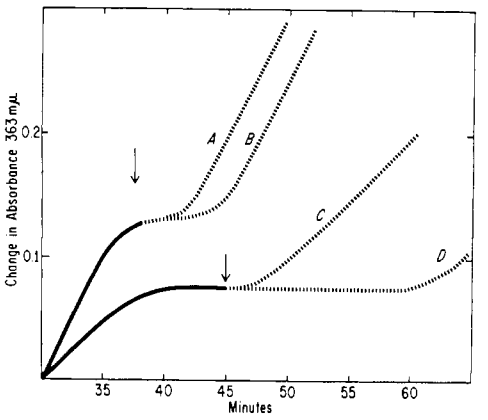

FIGURE 5: Kinetic curves illustrating the effect of substrate limitation and substrate depletion on various forms of the enzyme. Curve A represents the effect of the addition of further PP-ribose-P to enzyme initially added in state II′ form when this substrate is becoming limiting. Curve B is the result of a similar experiment with enzyme initially in state I or state II form. The results of a similar experiment when PP-ribose-P has been exhausted are shown with curve C (state II′ enzyme) and curve D (state I or state II enzyme). Note the time scale indicating that the velocities to the left are not initial velocities. Arrows indicate the point of substrate addition.

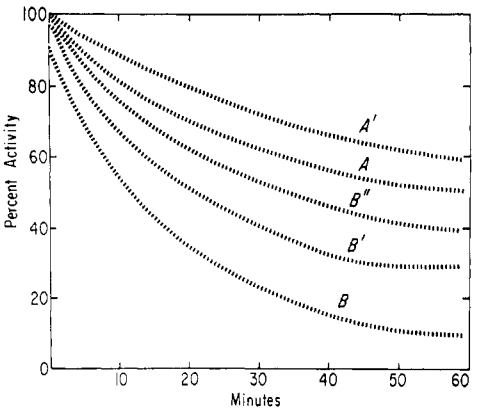

FIGURE 6: Inhibition of different forms of enzyme as a function of the time of incubation with OP (1.0×10^{-3} M) before assay. *A* represents state II′ enzyme, *B* state I enzyme, and *B″* state II enzyme. *B′* represents state I enzyme incubated with OP at a lower concentration of 5.0×10^{-4} M. *A′* represents state II′ enzyme incubated with OP (1.0×10^{-3} M) in the presence of PP-ribose-P (2.5×10^{-4} M) and Mg^{2+} (3.0×10^{-3} M).

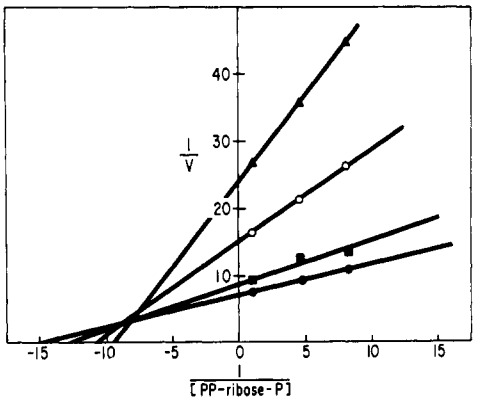

FIGURE 7: Lineweaver–Burk plot of PP-ribose-P with respect to OP. State I enzyme was used for the assays. All reagents were added at zero time, PP-ribose-P and Mg^{2+} being added immediately prior to OP. (●) No OP, (■) 1.0×10^{-4} M OP, (○) 5.0×10^{-4} M OP, and (▲) 1.0×10^{-3} M OP.

the same response was seen (Figure 5, curve C). With state I or state II enzyme, however, the lag was identical with that seen originally with the state I enzyme (Figure 5, curve D). This indicated a reversal of state III enzyme to the original state I form and supports the distinction between state II′ and state II enzyme.

The exhaustion of glutamine by the repeated addition of PP-ribose-P resulted, of course, in a complete cessation of the reaction. The subsequent addition of glutamine in the presence of continued high levels of PP-ribose-P and Mg^{2+} caused a prompt reaction with the usual lag before maximal velocity was attained, a pattern similar to that shown in Figure 5 (curve A).

These studies indicate that state I enzyme, converted into state II by PP-ribose-P and Mg^{2+}, and further activated to state III in the presence of glutamine, reverted to state II form when glutamine was depleted and to the original state I form when PP-ribose-P was exhausted. State II′, however, did not appear to require PP-ribose-P for maintenance of this particular state, but required all substrates for conversions into state III form. Reversion to the state II′ form occurred on depletion of glutamine.

The interpretations of these experiments were supported by results obtained when another technique was employed. Different forms of the enzyme were incubated with various combinations of substrates, passed rapidly through a Sephadex G-25 column (10.0 × 1.0 cm), and assayed. Sephadex chromatography removed the substrates from the enzyme and the results obtained were consistent with the data above. Removal of PP-ribose-P and Mg^{2+} from state II enzyme resulted in a reversal to state I form, whereas form state II′ enzyme was unaffected. Removal of *all* substrates from the state III enzyme resulted in a reversion to state I form or to state II′ form, depending upon the original form of enzyme utilized in the experiment.

Sensitivity to Iron Chelator. We have previously reported that part of the iron present in the amidotransferase is involved in the active site and that the enzyme is strongly inhibited by iron chelators such as OP.[1] This inhibition could be blocked by prior addition of the specific substrates PP-ribose-P and Mg^{2+} but not by glutamine. Furthermore, with the form of the enzyme employed in these earlier studies, AMP would protect from the effects of OP irrespective of whether the enzyme was sensitive to inhibition by the nucleotide (Rowe and Wyngaarden, 1968). These interactions have now been examined in more detail, utilizing different forms of the enzyme.

Inhibition by *o*-phenanthroline is complex and probably involves both binding to the critical iron atoms and removal of these atoms. In the light of our previous work, the major effect on the enzyme during the time covered by these experi-

[1] Abbreviations used: OP, 1,10-*o*-phenanthroline; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate.

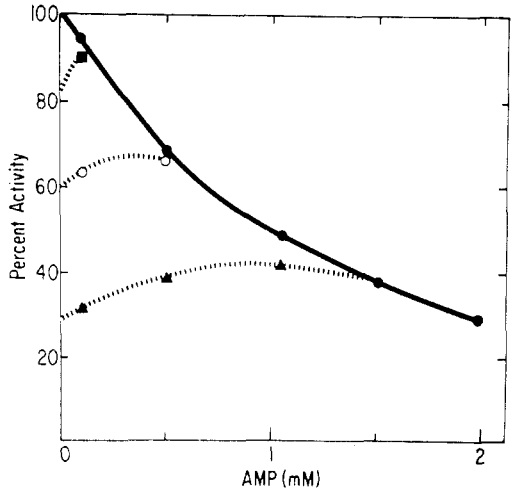

FIGURE 8: Protection of nucleotide-sensitive state I enzyme by AMP from the effect of OP. For these assays AMP in various concentrations together with 3.0×10^{-3} M Mg^{2+} was added to the enzyme 20 min prior to the addition of OP. The mixture was allowed to stand for a further 20 min prior to commencing the assay by the addition of the other required components. (●—○) No OP, (■) 1.0×10^{-4} M OP, (○) 5.0×10^{-4} M OP, and (▲) 1.0×10^{-3} M OP.

ments is probably related to steric hindrance resulting from binding of the bulky OP molecule to the iron (Rowe and Wyngaarden, 1968). The inhibition proceeds as a direct function of the time of exposure and of the concentration of the chelator (Figure 6, curves B and B′)—state I enzyme being more affected than state II′ enzyme (Figure 6, curves B and A).

Examination of the kinetics revealed a mixed competitive, noncompetitive inhibition of the enzyme by OP with respect to PP-ribose-P (Figure 7). This applied whether state II′, state I, or state II enzyme was studied. OP added during the course of the reaction when maximal velocity was attained showed no effect whatsoever, suggesting that the state III form of the protein was not susceptible to attack.

As mentioned above, the state I form of enzyme was extremely susceptible to the chelator, but conversion into

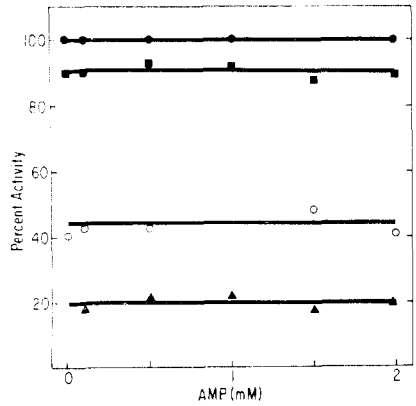

FIGURE 9: Failure of protection of nucleotide-insensitive state I enzyme from the effects of OP by the prior addition of AMP. (●) No OP, (■) 1.0×10^{-4} M OP, (○) 5.0×10^{-4} M OP, and (▲) 1.0×10^{-3} M OP.

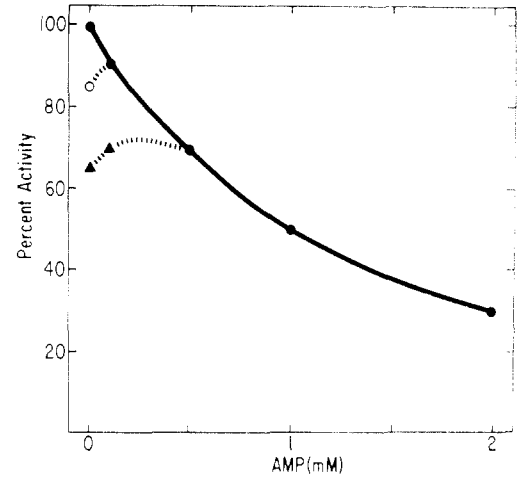

FIGURE 10: Protection of nucleotide-sensitive state II′ enzyme from OP by varying concentrations of AMP. (●) No OP, (○) 5.0×10^{-4} M OP, and (▲) 1.0×10^{-3} M OP.

state II by incubation with PP-ribose-P and Mg^{2+} decreased this sensitivity (Figure 6, curve B′). State II′ enzyme, already relatively resistant, was rendered even more resistant by addition of the specific substrates (Figure 6, curves A and A′).

State I enzyme, sensitive to inhibition by AMP, was partly protected from the effect of the chelator by the prior addition of the nucleotide together with Mg^{2+} (Figure 8). Little or no protection was seen if the Mg^{2+} was omitted, indicating a requirement for this cation in the binding of AMP. The activity curve for enzyme in the presence of varying concentrations of AMP became coincident with the combined AMP and OP curves at (1) 1.5×10^{-4} M AMP for 1.0×10^{-4} OP; (2) 6.0×10^{-4} M AMP for 5.0×0.10^{-4} M OP; and (3) 1.5×10^{-3} M AMP for 1.0×10^{-3} M OP. For clarity, the latter parts of the combined curves are omitted as they are coincident. If one reversed the order of addition of OP and AMP to the enzyme during preincubation, the inhibition produced was the equivalent of an *additive* effect of both inhibitors.

With state I enzyme insensitive to AMP, however, this protection is not seen (Figure 9). This experiment also confirmed that the protection by AMP was not merely the result of a relatively nonspecific effect, such as the binding of OP by AMP.

Additional protection of the state II enzyme was difficult to demonstrate because of the obligatory presence of PP-ribose-P and Mg^{2+} in the generation of this form of the enzyme.

State II′ enzyme was protected from OP by the presence of AMP whether or not it was sensitive to the nucleotide (Figures 10 and 11). This observation indicated that this form of the enzyme bound the nucleotide even though insensitive to its inhibitory effect. Figure 10 demonstrates again that state II′ enzyme is relatively insensitive to the chelator. The AMP inhibition curve becomes coincident with the combined curves at (1) 1.0×10^{-4} M AMP for 5.0×10^{-4} M OP, and (2) 5.0×10^{-4} M AMP for 1.0×10^{-3} M OP. The lowest concentration of OP used with state I enzyme (1.0×10^{-4} M) is not shown, as its inhibitory effect is minimal. With nucleotide-insensitive enzyme the same protection is also demon-

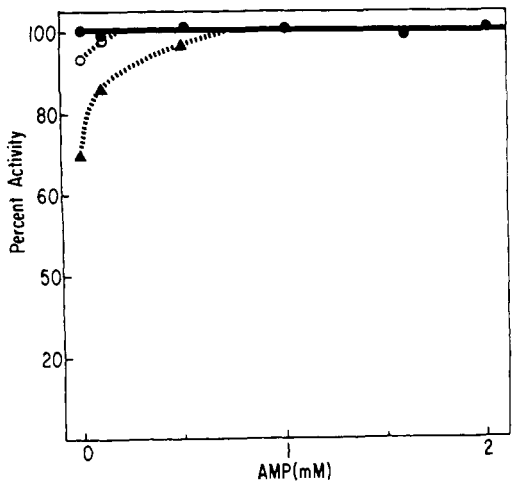

FIGURE 11: Protection of nucleotide-insensitive state II′ enzyme from OP by varying concentrations of AMP. (●) No OP, (○) 5.0×10^{-4} M OP, and (▲) 1.0×10^{-3} M OP.

strated (Figure 11), the curves becoming coincident at approximately the same AMP concentrations. Again, for clarity, coincident plots are omitted.

Determination of Kinetic Constants. Kinetic studies were carried out on all forms of the enzyme. As one might expect, when the reaction velocities used in the calculations were those obtained with state III enzyme, *i.e.*, at maximal velocity for the substrate concentration employed, the kinetic constants obtained from Lineweaver–Burk plots were identical for enzyme which had initially been in state I or II form. The state II′ form also exhibited similar values. As reported previously (Rowe and Wyngaarden, 1968), the values were slightly different from those reported in earlier work. The K_m values for glutamine (7.5×10^{-4} M) and Mg^{2+} (3.0×10^{-4} M) were similar, but the values were somewhat lower for PP-ribose-P (6.0×10^{-5} M).

Sigmoid kinetics were demonstrated (Figure 12), with sigmoidal relationships accentuated in the presence of the negative effector AMP. These kinetics applied to all nucleotide-sensitive forms of the enzyme. AMP did not affect the kinetic plot for insensitive enzyme, although minimal sigmoidicity was still demonstrable.

Fluorescence Studies with a Hydrophobic Probe. Different forms of the enzyme were examined with the fluorescent hydrophobic probe TNS, which exhibits virtually no fluorescence in water but fluoresces strongly in hydrophobic solvents or when bound to the hydrophobic regions of proteins (McClure and Edleman, 1966, 1967). In each case the enzyme was approximately 80–90% pure, as eluted from DEAE-cellulose in buffer containing thiol, and subsequently concentrated by ultrafiltration (Rowe and Wyngaarden, 1968).

When TNS was added to a solution of state I or state II′ enzyme, there was an immediate 100-fold increase in fluorescence with an emission peak maximal at 530 mμ (Figures 13 and 14). The prior addition of Mg^{2+} to the enzyme resulted in a 3-fold enhancement of the intensity of fluorescence produced in the absence of Mg^{2+}. Furthermore, there was a short blue spectral shift, the emission peak centered at 420 mμ (Figures 13 and 14).

With state I enzyme, glutamine or PP-ribose-P added, either alone or together (but without Mg^{2+}), failed to induce any fluorescence changes similar to those seen with Mg^{2+}. The changes seen with the simultaneous addition of Mg^{2+} and PP-ribose-P were no different from those seen with Mg^{2+} alone.

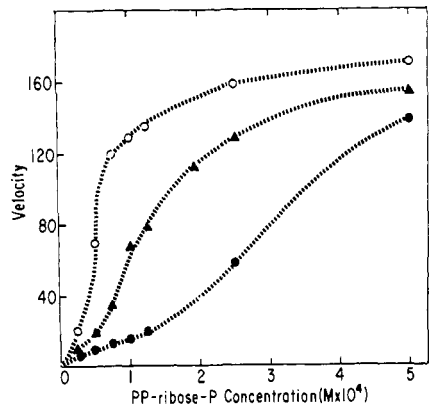

FIGURE 12: Michaelis–Menten plot illustrating the sigmoid kinetics with respect to PP-ribose-P. (○) No added AMP, (▲) 1.0×10^{-3} M AMP, and (●) 2×10^{-3} M AMP.

With state II′ enzyme, however, glutamine (in the presence of Mg^{2+}) induced an immediate additional 50% increase in fluorescence (Figure 13), the emission peak still being maximal at 420 mμ. No PP-ribose-P was required to demonstrate this effect.

By contrast, PP-ribose-P and Mg^{2+} were both required for glutamine to produce a similar effect with state I enzyme (Figure 14). In fact, the enhanced fluorescence was maximal when the glutamine was added 5–10 min after the other substrates. This effect could well be related to conformational changes. The changes induced by glutamine were extremely rapid, occurring virtually instantaneously.

The order of addition of reagents in these experiments is critical. The various substrates must be added to the enzyme before the fluorophore, otherwise no change in fluorescence occurs. This observation suggests that the various effectors influence the conformation of the enzyme molecule to expose

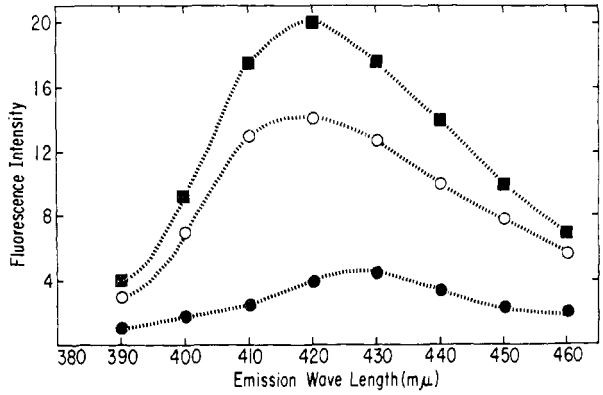

FIGURE 13: Fluorescence spectra of state II′ enzyme–TNS complex. Protein concentration, 0.52 mg/ml. TNS, 1.0×10^{-5} M. (●) Protein–TNS; (○) protein–TNS with Mg^{2+}, 3.0×10^{-3} M; (■) protein–TNS with Mg^{2+}, 3.0×10^{-3} M; and glutamine, 1.0×10^{-3} M. TNS is added last. Excitation wavelength 366 mμ.

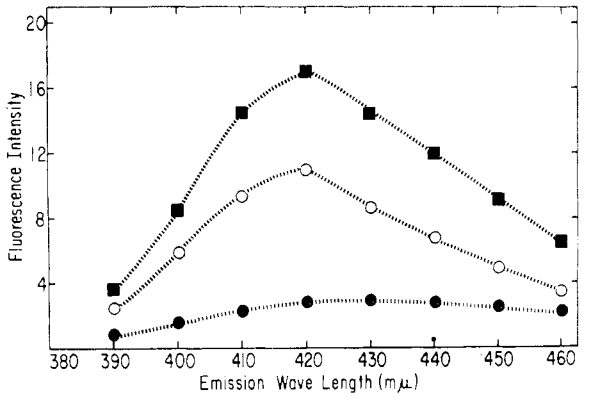

FIGURE 14: Fluorescence spectra of state I enzyme–TNS complex. Protein concentration 0.54 mg/ml of TNS (1.0×10^{-5} M). (●) Protein–TNS; (○) protein–TNS with Mg^{2+}, 3.0×10^{-3} M; (■) protein–TNS with Mg^{2+}, 3.0×10^{-3} M, PP-ribose-P, 2.5×10^{-4} M; and glutamine 1.0×10^{-3} M. TNS is added last. Excitation wavelength 366 mμ.

specific hydrophobic regions with high affinity for TNS, but that TNS once bound is relatively firmly held, and that even the creation of more hydrophobic sites by the sequential addition of substrates does not result in the displacement of TNS molecules onto these newly exposed sites. In view of the observation that TNS did not affect enzyme activity, irrespective of its order of addition with regard to the substrates, it was unlikely that it prevented substrate binding.

If enzyme was allowed to become inactive by standing at room temperature for 24 hr, Mg^{2+} still effected an increase in fluorescence of the TNS–enzyme complex. The addition of other substrates, however, failed to produce any further changes.

Discussion

Based upon data presented in this paper a theoretical model may be constructed for the relationship between the various forms of the enzyme (Figure 15). The state I form of the enzyme is inactive catalytically, but in the presence of the specific substrates PP-ribose-P and Mg^{2+} it is converted into a catalytically active form. The transition of the state I enzyme molecule to state II form is inhibited strongly by the iron chelators, implying that the iron moieties involved in the active site are in an exposed or accessible position in the state I molecule. Incubation of the state I molecule with PP-ribose-P and Mg^{2+} results in protection of these iron atoms, implying that the conformational change leading to activation to state II also renders the iron of the active site less accessible to chelators. AMP will block chelator effects in the state I enzyme provided the enzyme is sensitive to inhibition by the nucleotide. The nucleotide-insensitive enzyme either does not bind the nucleotide or the nucleotide binding site is in such a position that it cannot afford protection to the active site.

The state II enzyme becomes fully active only after the addition of glutamine. The progressive acceleration during the course of the reaction implies a cooperative effect created by the binding of this second substrate. The fully activated or state III molecule is totally resistant to chelators, indicative

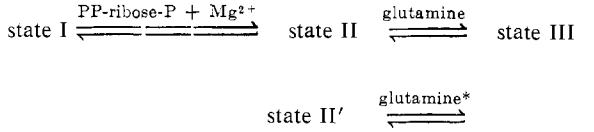

FIGURE 15: Model for relationships among various enzyme forms. (*) In the presence of PP-ribose-P + Mg^{2+}.

of a further conformational change rendering the active site inaccessible to the relatively bulky OP molecule.

Kinetic studies employing substrate exhaustion and substrate removal techniques indicate that the postulated conformational changes of the activation process are reversible. When levels of PP-ribose-P become limiting during the reaction, the enzyme, initially added in state I, reverts from state III to state II form, indicating the necessity of PP-ribose-P for the binding of glutamine. If PP-ribose-P is exhausted or totally removed from the reaction, a complete reversal to state I occurs. Removal or depletion of glutamine from fully activated (state III) enzyme causes a reversion to state II provided PP-ribose-P and Mg^{2+} are present. The reversal of the changes that characterize the activation process is accompanied by appropriate change in sensitivity to OP.

The state II′ form appears to be different from the state II form in that it does not require PP-ribose-P and Mg^{2+} for the maintenance of its activity, and removal of these substrates does not cause a transition to state I form. The enzyme is more resistant to OP and this resistance is always reinforced by AMP. This implies that, irrespective of the nucleotide inhibition phenomenon, the enzyme binds nucleotide and the conformation affords protection of the active site.

Occurrence of conformational changes is given further support by studies with the fluorescent hydrophobic probe. State I enzyme, under the influence of Mg^{2+}, undergoes a conformational change and exposes a more favorable hydrophobic environment for the binding of the fluorophore. For glutamine to induce any further changes, the protein molecule must be further modified by the addition of PP-ribose-P and Mg^{2+}.

State II′ enzyme also undergoes an instantaneous conformational change with the addition of Mg^{2+}. The further binding of glutamine with its associated conformational changes does not require the presence of PP-ribose-P.

The conformational changes induced by glutamine occur almost instantaneously, whereas the acceleration of the enzymatic reaction occurs over a period of several minutes. Perhaps these instantaneous changes are followed by as yet undetectable conformational changes more directly related to the active site and largely unrelated to those changes which are creating a more favorable hydrophobic environment for the fluorophore.

The possibility that the substrates alter the apparent binding constant for the TNS–enzyme complex has been excluded in the case of chymotrypsinogen and its various ligands (McClure and Edelman, 1967), and it not considered a likely explanation for the effects described here. This idea is reinforced by the obligatory order of addition of substrates and fluorophore required to observe an effect.

These studies demonstrate also that there is an ordered binding of the substrates. Standard kinetic studies which utilized the final maximum velocities attained were interpreted

initially in favor of a random-binding mechanism (Wyngaarden and Ashton, 1959). It now appears, however, that PP-ribose-P and Mg^{2+} are required for the subsequent binding of glutamine. Sequential binding has also been reported for the amidotransferase of Adenocarcinoma 755 cells (Hill and Bennett, 1969), and appears probable in the case of the chicken liver enzyme in which binding of PP-ribose-P and 6-diazo-5-oxo-L-norleucine is sequential (Hartman, 1963b).

We have observed that the purified enzyme loses all measurable activity in 72 hr, at $-20°$, and that during this process of decay state II′ enzyme is converted into state I enzyme. We reported in earlier work that the 200,000 mol wt enzyme dissociates into 100,000 mol wt dimer and 50,000 mol wt monomer subunits in the presence of 60 mM mercaptoethanol. The monomer behaves in the assay system as a state I form of the enzyme. However, we have not been able to demonstrate any reaggregation of subunits in the presence of various combinations of substrates. Thus it is not known presently whether transitions of state I to state II to state III forms involve changes in molecular weight of the enzyme.

Most new enzyme preparations in a high degree of purity behave as a mixture of state II′ and state I molecules, *i.e.*, there is a definite initial velocity but a prolonged lag prior to attaining maximal velocity. One might expect these findings under conditions where there is a mixed population of molecules of different states of aggregation if the kinetic properties are directly related to the degree of subunit association. Subunit association, however, does not necessarily imply activation of an enzyme (Stadtman, 1966), and perhaps the conformational changes are more subtle, lying within the monomer itself, as envisioned in the Koshland–Neet (1968) model of allosteric protein molecules.

The sigmoid kinetics indicate a higher order reaction with the specific substrates PP-ribose-P and Mg^{2+}, and this sigmoidicity is accentuated by the competitive allosteric inhibitor AMP. The apparent reversion toward hyperbolic unimolecular substrate kinetics in the presence of decreasing concentrations of the negative effector is attributable to a shift and compression of the sigmoid response curve along the substrate axis from high to low substrate concentrations. This is analogous to the situation reported by Atkinson and Walton (1965), with phosphofructokinase.

The multiple cooperative interactions of the amidotransferase with effector molecules may be summarized as follows: state I enzyme is modified by PP-ribose-P and Mg^{2+}; state II and II′ enzymes are modified by glutamine; state III enzyme shows additional cooperative effects with PP-ribose-P, manifested by sigmoid velocity–substrate curves. The plot of $\log v/(V - v)$ *vs.* $\log$ (S) gives a maximal slope of 1 (Caskey *et al.*, 1964); state III enzyme also shows cooperative interactions with negative effectors, manifested in two ways. Plots of $(V_0/V_1) - 1$ *vs.* $\log$ (I) for individual ribonucleotide inhibitors are sigmoid with maximal slopes >2, and combinations of 6-amino- and 6-hydroxypurine ribonucleotide inhibitors produce effects that are more than additive (Caskey *et al.*, 1964).

The type of catalytic and conformational heterogeneity described here for glutamine PP-ribose-P amidotransferase of pigeon liver is similar to that described for glutamine synthetase of *Escherichia coli*. In a series of elegant studies by Shapiro and Ginsburg (1968) and Kingdon *et al.* (1968), three forms of the enzyme were described, designated relaxed, tightened, and taut forms. Small differences in sedimentation constants and intrinsic viscosities were described among these forms, but transitions did not involve changes of molecular weight, or rearrangements of subunits. It would appear that the final conformational state of the protein is the sum of a series of complementary interactions between the protein and its ligands.

As yet we have not probed deeply into the physical changes in the amidotransferase molecule but there are obvious implications for the intracellular control of amidotransferase activity based upon regulation of kinetic properties by positive and negative effectors. Not only is the first enzyme of the purine biosynthetic pathway subject to allosteric inhibition by the end products of the pathway, but the intrinsic activity of the protein molecule is modulated by the availability of substrates which determine its conformational state.

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References

Atkinson, D. E., and Walton, G. M. J. (1965), *J. Biol. Chem. 240*, 757.

Caskey, C. T., Ashton, D. M., and Wyngaarden, J. B. (1964), *J. Biol. Chem. 239*, 2570.

Hartman, S. C. (1963a), *J. Biol. Chem. 238*, 3024.

Hartman, S. C. (1963b), *J. Biol. Chem. 238*, 3036.

Hill, D. L., and Bennett, L. L., Jr. (1969), *Biochemistry 8*, 122.

Kingdon, H. S., Hubbard, J. S., and Stadtman, E. R. (1968), *Biochemistry 7*, 2136.

Koshland, D. E., Jr., and Neet, K. (1968), *Ann. Rev. Biochem. 37*, 359.

McClure, W. O., and Edelman, G. M. (1966), *Biochemistry 5*, 1908.

McClure, W. O., and Edelman, G. M. (1967), *Biochemistry 6*, 559.

Nierlich, D. P., and Magasanik, B. (1965), *J. Biol. Chem. 240*, 358.

Rowe, P. B., and Wyngaarden, J. B. (1968), *J. Biol. Chem. 243*, 6373.

Shapiro, B. M., and Ginsburg, A. (1968), *Biochemistry 7*, 2153.

Stadtman, E. R. (1966), *Advan. Enzymol. 28*, 41.

Wyngaarden, J. B., and Ashton, D. M. (1959), *J. Biol. Chem. 234*, 1492.