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Biochemistry

Acetate kinase: A triple-displacement enzyme

(phosphoenzyme/covalent catalysis/adenine phosphoribosyltransferase/hexokinase/nucleosidediphosphate kinase activity)

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ABSTRACT **Facts relating to the mechanism of phosphoryl transfer by acetate kinase (ATP:acetate phosphotransferase, EC 2.7.2.1) are reviewed. They point to the existence of at least one experimentally established phosphoenzyme (E-*P*) intermediate on the reaction pathway. Sterically, the phosphoryl transfer occurs with a net inversion of the configuration of the phosphorus atom. These facts are best in accord with a triple-displacement mode of action for acetate kinase, with two E-*P* intermediates and three steric inversions on phosphorus. It follows that a second E-*P* for acetate kinase must exist.**

Ever since its discovery by Lipmann (1), acetate kinase (EC 2.7.2.1) has engaged the active interest of students of enzyme mechanism, and never more so than now. The enzyme catalyzes the synthesis of ATP from acetyl-*P*.

$$\text{Acetyl-}P + \text{MgADP} \overset{K^+}{\rightleftharpoons} \text{MgATP} + \text{acetate} \qquad [1]$$

In the reverse direction the enzyme is the prototypic carboxylate kinase. As such, the chemical means by which acetate kinase effects phosphoryl transfer has been much studied, with broad implications in the wider field of phosphotransferases generally.

REVIEW OF AVAILABLE DATA

The equilibrium constant of reaction 1, as catalyzed by the *Escherichia coli* enzyme, is reported to range from 200 to 1500 (1–5), indicating that, thermodynamically, the reaction greatly favors ATP synthesis from acetyl-*P*. In its specificity, the enzyme is most active on acetyl-*P* but has some activity on propionyl-*P* (30%) and carbamoyl-*P* (18%), and none on butyryl-*P*, glutaryl-*P*, and phospho*enol*pyruvate (4). The purines GTP, ITP, and dATP, as well as ATP, are excellent substrates (2, 4), accounting thus for the intrinsic purine nucleoside-5′-diphosphate kinase (NDP kinase, EC 2.7.4.6) activity of acetate kinase (6, 7).

It is curious that the K_m for acetate is 300 mM (2), one of the highest Michaelis constants on record. Another curiosity is the cold inactivation to which acetate kinase is liable (8). Chilled at 0°C, the enzyme quickly loses activity. Restoration of activity is possible; it is most rapidly (2–3 min) achieved, even at 0°C, by the addition of nucleotide (4). Acetate and acetyl-*P* are without effect. Reactivation in the absence of nucleotide requires a long-term (1–2 hr) incubation at room temperature in the presence of Mg^{2+} and a monovalent cation (7, 8). If these inactivation–reactivation phenomena are due to conformational changes in the protein, as seems probable, then it is clear that only the nucleotides, of the four substrates of reaction 1, can specifically induce the active conformation of the enzyme.

Phosphorylation of Acetate Kinase. Fig. 1 shows that the enzyme can be phosphorylated with ATP or acetyl-*P*, and the phosphoenzyme (E-*P*) can be isolated by gel filtration (6, 9, 10). The E-*P* prepared with ATP and the E-*P* prepared with acetyl-*P* are identical with respect to pH-stability, sensitivity to hydroxylamine, and the capacity to phosphorylate ADP or acetate (4). The phosphoryl group in E-*P* is linked to a carboxyl of the enzyme (9). Reduction of E-*P* with boro[^{3}H]hydride (and isolation, after hydrolysis, of tritiated α-amino-δ-hydroxyvalerate) reveals that the γ-carboxyl of a glutamyl residue is in fact the site of phosphorylation (11). For undetermined technical reasons, the extent of phosphorylation of the enzyme is found to be quite variable (9). Nevertheless, a close parallel exists between the degree of reducibility of an E-*P* preparation and its degree of phosphorylation (10), showing that the different E-*P* preparations are chemically identical.

Chemical and Kinetic Competence of E-*P*. E-*P* can transfer its phosphoryl group quantitatively to ADP within 1 min (the earliest time point taken), in the absence of cosubstrates (8). The same transfer can be made to GDP (6). The quantitative synthesis of ATP from E-*P* is of course in the thermodynamically "downhill" direction of reaction 1. Although no direct evidence for the kinetic competence of E-*P* in ATP synthesis is available, such competence seems nonetheless very probable. I venture the guess that if the reaction of E-*P* with ADP were to be studied in a stopped-flow apparatus, a short transfer time would be found. Some indirect evidence for the kinetic competence of E-*P* in ATP synthesis is offered below in connection with the ADP–ATP exchange.

In further proof of its chemical competence, E-*P* can phosphorylate acetate, in the absence of added nucleotide, in the thermodynamically "uphill" direction of reaction 1. Here the phosphorylation is slower and less extensive, a 70% yield of acetyl-*P* being possible in 15 min (9). In the direction of acetyl-*P* synthesis it seems less meaningful to speak of the kinetic competence of E-*P* because, in the absence of nucleotide, the E-*P* is not in its optimal conformation for activity.

ADP–ATP Exchange. This reaction is catalyzed by acetate kinase in the virtually certain absence of the cosubstrates, acetate and acetyl-*P*. Adventitious acetate, with its high K_m, or acetyl-*P*, with its sensitivity to hydrolysis, cannot survive the several steps of enzyme purification. The ADP–ATP exchange reaction is therefore independent of cosubstrates. Nor can it be imputed to a contaminating enzyme. Fig. 2 shows that during gel electrophoresis the exchange activity of the enzyme migrates precisely in step with its kinase activity (8). Moreover, it is clear from Fig. 3 that the ADP–ATP exchange is unaffected by acetate, which is added in order to institute the acetate kinase activity; or, otherwise put, the ADP–ATP exchange and the kinase reaction proceed at the same rate (5, 8). It would be an extraordinary coincidence if the exchange, were it catalyzed by a contaminating enzyme, had precisely the same rate as the kinase reaction catalyzed by acetate kinase. This, conjoined with the coincidence of exchange and kinase activities on gel elec-

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Abbreviations: E-*P*, phosphoenzyme; NDP kinase, purine nucleoside-5′-diphosphate kinase.

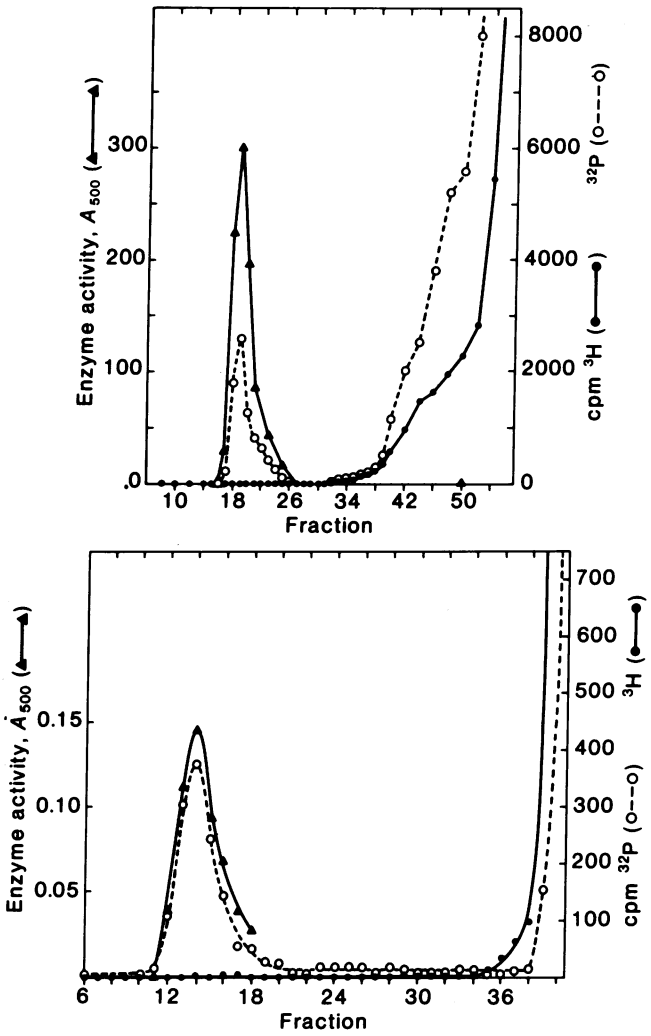

FIG. 1. Sephadex G-25 gel filtration of acetate kinase phosphorylated with [^{3}H,γ-^{32}P]ATP (*Upper*) or [^{3}H,^{32}P]acetyl-*P* (*Lower*). After incubation and gel filtration, small samples of the fractions were taken for measurement of radioactivity and kinase activity, which was assayed by the hydroxamate procedure (8). Reproduced with permission (9).

trophoresis (Fig. 2), makes it a near certainty that the ADP–ATP exchange is intrinsic to acetate kinase.

Further to the same point is the observation that E-*P* (prepared with acetyl-*P*) loses kinase activity upon exposure to hydroxylamine, as the catalytic carboxyl in the active center is transformed into hydroxamate. It is found that the rate of loss of kinase activity parallels closely the rate of loss of ADP–ATP exchange activity (10). An intimate linkage is thus established between the two activities centering on a catalytic carboxyl group.

In this connection, some experiments made on the acetate kinase of *Veillonella alcalescens* are in nice accord with those made on the *E. coli* enzyme. The former enzyme can be inhibited with iodoacetate (12). When 1 mol of inhibitor is covalently fixed to each of the two subunits of the enzyme, both the kinase and the ADP–ATP exchange activities are completely suppressed. Alkali treatment of the inhibited enzyme releases glycolic acid, implying ester formation between iodoacetate and a carboxyl in the active center. Here again, we have evidence of a close tie between the kinase and exchange activities through an enzyme-carboxyl.

Returning to the question of the kinetic competence of E-*P* in the overall kinase reaction, we note again from Fig. 3 that

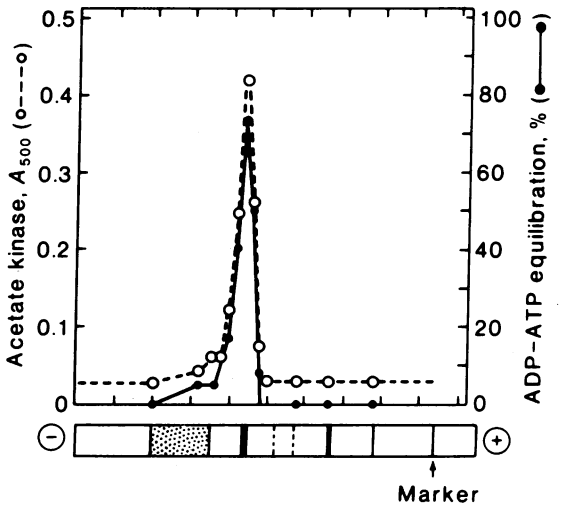

FIG. 2. Coincidence of acetate kinase and ADP–ATP exchange activities in gel electrophoresis. Disc gel electrophoresis of the enzyme was conducted in duplicate. One gel was stained with Coomassie blue and is diagrammed at the bottom of the figure (migration of the protein was from left to right). Superposed above it are the profiles of acetate kinase and ADP–ATP exchange activity, as determined with the duplicate gel, which was sectioned. Reproduced with permission (8).

the rate of ATP synthesis is the same in the exchange and in the kinase reactions. It follows that E-*P*, a component of the exchange and chemically competent in the kinase reaction, is also kinetically competent in the latter reaction.

Acetate–Acetyl-*P* Exchange. Acetate kinase also catalyzes the incorporation of acetate into acetyl-*P* in the absence of added nucleotide (8). The rate of the exchange is slow, the ADP–ATP exchange being several hundredfold faster. This is only natural, because nucleotide is essential for optimal enzyme conformation. And, because the enzyme's endogenous content of nucleotide is unknown, it cannot be claimed that the acetate–acetyl-*P* exchange is truly independent of cosubstrates (5). For this reason, little weight is given to it as an argument for covalent catalysis by acetate kinase.

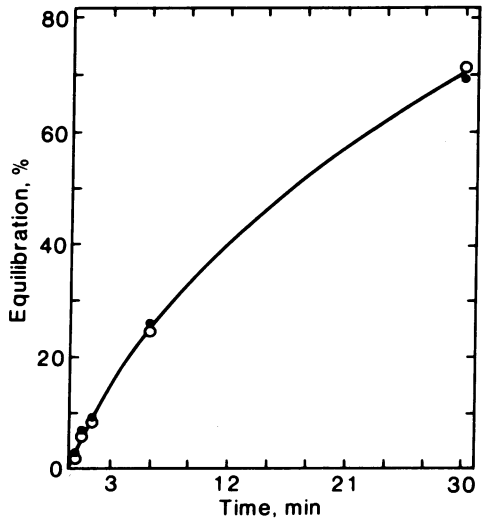

FIG. 3. Rate of incorporation of [8-^{14}C]ADP into ATP as catalyzed by acetate kinase in the presence (•) and absence (O) of acetate. The two incubated solutions were identical except for the addition of acetate to one of them in order to institute the overall reaction. The rates of [8-^{14}C]ATP formation are seen to be the same. Reproduced with permission (8).

Purine NDP Kinase Activity of Acetate Kinase. Somewhat in the same vein as the experiments measuring the ADP–ATP exchange are those made on the purine NDP kinase activity of the enzyme. Because it is active on GTP and GDP, as well as ATP and ADP, the enzyme can catalyze a reversible phosphoryl transfer between GTP and ADP, in the manner of the classical NDP kinase (13). Like the ADP–ATP exchange, the NDP kinase activity is intrinsic to acetate kinase. Thus, when E-*P* (prepared with acetyl-*P*) is exposed to hydroxylamine, the acetate kinase and NDP kinase activities are lost at the same rate (7). The NDP kinase reaction catalyzed by acetate kinase has, moreover, a kinetic mechanism that is authentic "ping pong." The *chemical* mechanism of the reaction is therefore a double displacement, proceeding over an E-*P* intermediate that is kinetically important (7). This is especially significant because the overall acetate kinase reaction has a kinetic mechanism that is *sequential* (10). With the same E-*P* on the pathway of both reactions, it is plain that covalent catalysis by acetate kinase is compatible with either "ping pong" or sequential kinetics (14).

Inhibitions by Mercuric Ion. Of peculiar pertinence to the chemical mechanism of acetate kinase is the remarkable effect of Hg^{2+} on the enzyme and its reactions (Table 1). At 10 μM Hg^{2+}, the overall acetate kinase reaction and the acetate–acetyl-*P* exchange are 90% or more inhibited. Yet, even at a 33-fold higher concentration of Hg^{2+}, the ADP–ATP exchange remains unimpaired (8). In perfect harmony with the above is the effect of Hg^{2+} on the reaction of E-*P* with acetate and ADP. The reaction of E-*P* with acetate is blocked totally by Hg^{2+}. But the quantitative synthesis of ATP from E-*P* and ADP is entirely immune to the influence of Hg^{2+} (9). We have thus a mercurated enzyme that *cannot* form E-*P* reversibly from acetyl-*P*, even in the presence of enzyme-activating nucleotide (in the overall reaction). But it *can* form E-*P* reversibly from ATP, with an ease equal to that of the normal enzyme (Table 1). The nucleotide subregion of the enzyme is evidently as active in the mercurated enzyme as it is in the normal.

These facts argue, at the very least, for a separation in space of the nucleotide and acetate (and acetyl-*P*) subregions of the active center. The separation must be sufficiently large to admit of a disruption by Hg^{2+} of the normal catalytic events in the acetate (and acetyl-*P*) region, while leaving unperturbed the events normal to the nucleotide region. Were the two regions contiguous in the active enzyme, a disturbance in one of them could hardly escape some degree of transmission to the other. But not the slightest disturbance is transmitted. There is a total disruption of activity in one region and a total failure to disrupt in the other. If the regions are spatially separated, as they seem thus to be, then *direct transfer of a phosphoryl group between donor and acceptor, in a single displacement, is rendered unlikely.* Best fit to bridge the gap might be a catalytic group (or two) in the active center of acetate kinase. This calls to mind a picture of the phosphoryl group of E-*P*, at the γ-carboxyl end of a glutamyl residue, swinging between one subregion and the other of the active center.

Stereochemical Inversion on Phosphorus During Kinase Activity. Of the highest importance to ascertaining the chemical mechanism of acetate kinase is knowledge of the stereochemical course of the reaction. Such knowledge has long been awaited by all who have an interest in this enzyme and in kinases generally. Now we know, from the elegant work of Blättler and Knowles (15), that the acetate kinase reaction proceeds with net steric *inversion* on the transferring phosphorus atom.

Table 1. Mercuric ion inhibitions

Reaction	$HgCl_2$, μM	Inhibition, %
Overall	11	90
Acetate–acetyl-*P* exchange	11	93
ADP–ATP exchange	330	0

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DISCUSSION

From an assessment of all the available data we think it right to conclude that the acetate kinase reaction proceeds by a pathway that includes a phosphoenzyme intermediate. This paramount fact excludes the single displacement. There are, moreover, no *unambiguous* data in support of it. But the finding of a net steric inversion in a reaction involving an E-*P* intermediate poses a profound problem of chemical mechanism. The inversion at phosphorus has been construed as resulting from a direct transfer of phosphoryl from donor to acceptor (15), but such is plainly incompatible with the E-*P* intermediate. Thus it is that the inversion at phosphorus and the E-*P* intermediate form a pair of firmly based but seemingly discordant facts. And it remains therefore to reconcile them.

Extensive investigations into the chemistry of tetra- and pentacoordinate phosphorus make it extremely probable that all enzymic substitutions at phosphorus involve in-line (S_N2-type) displacements with steric inversions (15–17). It follows that an enzyme with a single E-*P* on its pathway ought to catalyze a net *retention* of configuration on phosphorus. Thus, the inversion incurred during E-*P* formation should be reversed by a second inversion as E-*P* reacts with acceptor, the two inversions resulting in net retention. And, indeed, such net retentions have been found for galactose-1-*P* uridylyltransferase (EC 2.7.7.10) (17), the classical NDP kinase (17), alkaline phosphatase (EC 3.1.3.1) (18), and phosphodiesterase I (EC 3.1.4.1) (19, 20), all of which have a double-displacement mechanism with a demonstrated E-*P* on the reaction pathway.* But acetate kinase with an E-*P* on its pathway catalyzes not a retention but a net *inversion* on phosphorus. Nothing of what we know about the detailed substitution chemistry at phosphorus tells us why acetate kinase, in its steric action, should differ from the above-cited enzymes. There is no valid reason to suppose, for instance, that pseudorotation of a pentacoordinate intermediate should intervene in the action of acetate kinase but not in that of the other enzymes. There is, on the contrary, every reason to invoke in-line displacements at phosphorus for all these enzymes and, indeed, for phosphotransferases generally (15–17). On this view it becomes proper to think of a triple-displacement mechanism for acetate kinase, with two E-*P* intermediates and three inversions on phosphorus (Fig. 4).

Acetate kinase is not the first to reveal itself as a triple-displacement enzyme. Adenine phosphoribosyltransferase (EC 2.4.2.7) catalyzes the synthesis of AMP from 5-*P*-ribosyl-1-pyrophosphate in a reaction involving a net steric inversion on carbon-1 of the ribose ring (Fig. 5). A phosphoribosyl-enzyme is an intermediate of the reaction. The enzyme is in fact isolated from tissue as the phosphoribosyl-enzyme (21). A triple-displacement process, with two phosphoribosyl-enzyme intermediates and three S_N2 reactions (Walden inversions) on carbon-1 offers the best accounting for these facts.†

Orotate phosphoribosyltransferase (EC 2.4.2.10) catalyzes a reaction chemically analogous to the one of Fig. 5, with the

* In some of these enzymes the *P* in E-*P* is a substituted phosphoryl group.

† Substitutions at carbon, unlike those at phosphorus, are untroubled by questions of pseudorotation.

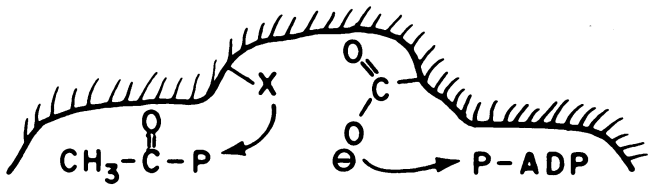


FIG. 4. Representation of the active center of acetate kinase. Associated with the nucleotide-binding region is the catalytic carboxyl that has ATP as its immediate phosphoryl donor. And associated with the acetate (and acetyl-*P*) binding region is an unknown group (X) that has acetyl-*P* as its immediate phosphoryl donor. The phosphoryl group is reversibly transferable between X and the carboxyl group. All phosphoryl transfers among substrates and the catalytic groups proceed with steric inversion on the phosphorus. It is conjectured that the intermediate E-X-PO_3 is at a higher energy level than E-COO-PO_3, because only the latter is isolated when the enzyme is phosphorylated by ATP or acetyl-*P*.

base orotate replacing adenine in the chemical structures. In this case, the phosphoribosyl-enzyme has not been isolated; but the enzyme follows perfect BiBi "ping pong" kinetics and catalyzes the appropriate exchanges, making the phosphoribosyl-enzyme an all but certain component of the reaction (22). Again, the inversion on carbon and the covalent intermediate find their best rationale in a triple displacement.‡

Hexokinase (EC 2.7.1.1) is another enzyme that shows signs of triple-displacement behavior. It catalyzes the transfer of a phosphoryl group from ATP to glucose with a net inversion at phosphorus (15). Its catalysis of genuine (but slow) ADP–ATP and glucose–glucose-6-*P* exchanges (in the assured absence of cosubstrates) intimates a role for an E-*P* in these reactions (23).§ In the presence of inhibitory D-xylose, hexokinase can be ("lethally") phosphorylated by ATP (24). *The E-P, so prepared, is chemically competent* in the sense that it can reverse its own synthesis by making ATP from ADP. But it cannot act on glucose to make glucose-6-*P*. The aggregate data suggest an active center for hexokinase much like the one of Fig. 4 for acetate kinase, with two catalytic sites, one of which has ATP for its immediate phosphoryl donor. In normal action the phosphoryl is further transferred to a second site, and from thence to glucose. The three phosphoryl transfers, occurring each with inversion, yield a net inversion for the total reaction.

‡ Whether the phosphoribosyl group is linked to the enzyme by a genuine covalent bond or by an "ion pair" bond is, from the standpoint of covalent catalysis, largely a question of semantics.

§ The speed of these exchanges in the absence of cosubstrates is inherently substantial. It is slow relative to the overall reaction, which is quite rapid. It is known from crystallography and other means that the presence of both substrates (i.e., ATP and glucose) is necessary to optimize the enzyme's conformation for reaction. It is only natural to suppose that in the exchange reactions (with cosubstrates absent) the conformation of the enzyme is suboptimal and that activity is impaired. It is the more remarkable that independent exchange activity occurs at all.

In accord with these notions are the recent crystallographic studies made on hexokinase and its substrates. A large conformational change occurs in the enzyme upon binding of glucose, with concurrent closure of the active site cleft around the glucose molecule. When ATP is placed in its most probable position in the model of the enzyme–glucose complex it is found that the γ-phosphoryl of ATP is at a nonbonding distance (5.5 Å) from the 6-hydroxyl of glucose (25). The available x-ray data thus imply that a direct transfer of phosphoryl from ATP to glucose is *not* possible.

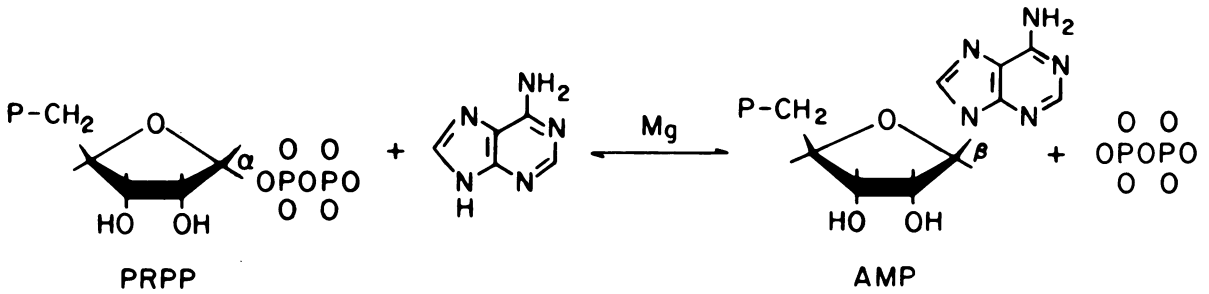
The existence of a spatial gap between the nucleotide and glucose (and glucose-6-*P*) binding regions of hexokinase is independently suggested by the effect of *N*-acetylglucosamine on the enzyme. *N*-Acetylglucosamine is a structural analogue of glucose and binds in the glucose site of hexokinase. In so doing, it inhibits the overall reaction but has no effect on the intrinsic ADP–ATP exchange activity that the enzyme possesses (26). These findings recall the above-noted effects of mercuric ion on the activities of acetate kinase. They hint strongly at a separation between the phosphoryl donor and acceptor regions of hexokinase that is sufficiently wide to permit disruption of chemical events in the acceptor region while leaving unperturbed the events in the donor region.

One means by which an enzyme could speed up a chemical reaction is to bind its donor and acceptor substrates in noncontiguous regions of the active center, and then use covalent catalysis to span the gap. Noncontiguous binding of substrates avoids the "three-body collision" that the single displacement imposes upon its substrates—that is, two substrates are forced into optimal contact and alignment with each other (for direct transfer) and with a specific locus in the active center. A three-body collision necessarily imposes a high degree of order, which is costly in activation energy. Covalent catalysis, by contrast, makes use of a suite of "two-body collisions," in which one of the two bodies is the catalytic group of the enzyme or the catalytic group with substrate fragment attached. Two-body collisions are of course far more probable than the three-body kind, and are therefore entropically favored.

An enthalpic benefit can also accrue from covalent catalysis. An enzyme must do work to force two atoms into close enough contact to form a covalent bond between them. Such work must surely be easier for the enzyme when one of the two atoms being forced into contact is a part of the enzyme itself than when both of the atoms are external to the enzyme; much as to make contact with a football is easier with one's own foot than with someone else's foot.¶

In the long history of enzymology no unambiguous evidence for the single-displacement mechanism has ever been given for *any* enzyme (14). By contrast, positive evidence for covalent

¶ This is intuitively obvious, but as a piece of anthropomorphism it is not easily explained in physical terms.



PRPP AMP

FIG. 5. Reaction catalyzed by adenine phosphoribosyltransferase, showing the steric inversion that occurs on carbon-1 of the ribose ring from the α configuration in phosphoribosylpyrophosphate (PRPP) to the β configuration in AMP.

catalysis is available now for over 400 enzymes, embracing in their number virtually the entire range of the enzyme classes and sub-classes (unpublished compilation). In place of the "simple elegance" of the single displacement, enzymes choose the seemingly greater complexity of covalent catalysis. With itself as a covalent partner the enzyme can divide its total reaction into a suite of partial reactions, and find therewith an energetically easier pathway. Nowhere is this better exemplified than in the case of acetate kinase.

One can only guess at why acetate kinase should need the two E-*P*s to consummate its reaction. Possibly the space between donor and acceptor is too wide to be bridged by one E-*P*. Or, even if the space is not too wide, the acceptor in its binding site may be so oriented as to need a second E-*P* to bring the phosphoryl group in from the best direction. In that case, the itinerant phosphoryl would not traverse a straight route between donor and acceptor, but a roundabout one.

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