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PYRIDOXAL PHOSPHATE IS NOT THE ACID CATALYST IN THE GLYCOGEN PHOSPHORYLASE CATALYTIC MECHANISM

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SUMMARY. Pyridoxal-reconstituted phosphorylase has been shown previously (Parrish, R.F., Uhing, R.J. and Graves, D.J. (1977), Biochemistry 16, 4824) to be activated to a very similar extent by phosphite (pK_a=6.6) and fluorophosphate (pK_a=4.8). This paper shows that the pH dependence of the enzyme activity is also similar for these two activators. This is interpreted to mean that the coenzyme phosphate is not involved as an essential acid catalyst in the actual reaction mechanism and is probably not involved in any form of proton transfer process. The pH/activity profile for native phosphorylase b in the direction of glycogen synthesis is also presented and a comparison of the two sets of profiles is discussed.

The role of PLP[1] in the catalytic mechanism of glycogen phosphorylase has been under investigation for many years now (see Helmreich & Klein (1980) (1) for a recent review), but no definitive conclusions have yet been attained. Several of the roles proposed have involved the phosphate of PLP in some kind of proton transfer process, either as an acid (2, discussed in 3) or as a base (4). The role of PLP as a base deprotonating the 4-hydroxyl of the oligosaccharide has been the most widely received of late on the basis of the ^{31}P nmr studies of Helmreich's group (4,5,6). Their assignment of the active form of the coenzyme phosphate as a mobile dianion has been cast into doubt recently on the basis of ^{31}P nmr studies of an "activated" ternary complex (7). This study concluded that the active form of the coenzyme phosphate is either the monoanionic form or a tightly liganded and possibly distorted dianionic form. In order to resolve this dilemma it was felt that an appropriate study of the pH dependence of enzyme activity could help. Several studies have been performed

[1]Abbreviations: PLP = pyridoxal phosphate.
PL-phosphorylase = phosphorylase b reconstituted with pyridoxal.

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previously on the effects of pH on the kinetic parameters for phosphorylase (8,9,10,11), the most recent of these (11), having concluded that the acid limb of the pH profile determined in the direction of glycogenolysis, $pK_a \simeq 6.0$ represents the ionisation of the coenzyme phosphate. This would imply an involvement of the phosphate of PLP as a base in the reaction mechanism; however, this assignment was made rather speculatively. A study of the effects of pH on the kinetics of pyridoxal-reconstituted phosphorylase b should yield information on the potential involvement of the coenzyme phosphate in a proton transfer process since this form of phosphorylase is active only in the presence of phosphate or a suitable analogue such as phosphite or fluorophosphate (12). It is believed that these phosphate analogues activate the enzyme by occupying the vacant phosphate binding site adjacent to the pyridoxal ring, and acting in its stead. Moreover, these two phosphate analogues show almost identical activation parameters (Vm=10.2 $\mu mol.min^{-1}.mg^{-1}$, Km app.=0.9 mM for phosphite; Vm=9.7 $\mu mol.min.^{-1}.mg^{-1}$, Km app.=2.0 mM for fluorophosphate). This is somewhat surprising in view of the substantial differences in their electronic properties (pK_a's=6.6 and 4.8 for phosphite and fluorophosphate respectively) (12). A complete determination of the pH dependence of the kinetic parameters of PL-phosphorylase b in the presence of each activator should therefore indicate whether such a proton transfer process is occurring, since an involvement of the coenzyme phosphate moiety in such a process would have to be reflected in substantially different pH profiles for the two different activators, when the profile for fluorophosphate should be shifted down almost 2 pH units in one of its limbs relative to that for phosphite. Such a direct comparison of two pH profiles in essentially the same enzyme system should eliminate many of the difficulties inherent in the normal interpretations of pH/rate profiles as well expressed previously by Knowles (13).

MATERIALS AND METHODS

Rabbit muscle phosphorylase b was prepared by the method of Fisher and Krebs (14) using DTT instead of cysteine, and recrystallized at least three times before use. Apo-phosphorylase b was prepared by resolution of phosphorylase b as described previously (15), and immediately reconstituted with a 50-fold excess of pyridoxal to produce PL-phosphorylase b. Such PL-phosphorylase b was

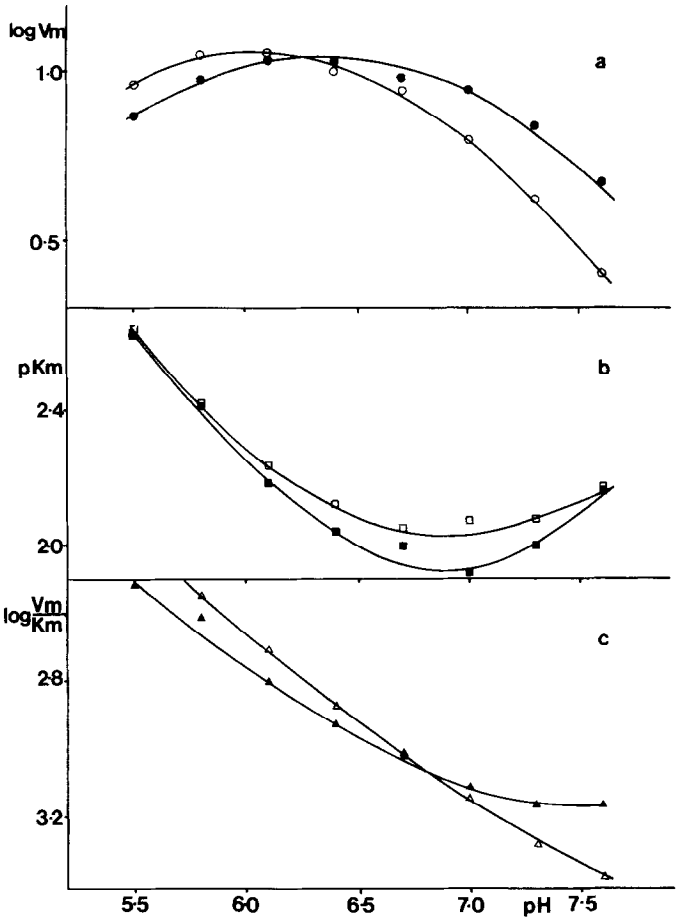

Figure 1. pH dependence of kinetic parameters for pyridoxal-phosphorylase b in the presence of (●) phosphite and (○) fluorophosphate; glucose-1-P as variable substrate in range 10 to 61 mM. Reactions performed in presence of 1 mM AMP, 1% glycogen and 7.5 mM phosphite or fluorophosphate.

used for kinetic studies over a three day period and then discarded. Initial reaction rates were determined at 30°C by the Fiske-Subarrow phosphate analysis in the direction of glycogen synthesis as described by Engers et al (16). Concentrations of substrates and activators employed in each case are as described in the appropriate figure legends. The buffer used in all these kinetic experiments was 50 mM triethanolamine hydrochloride, 100 mM potassium chloride, 1 mM DTT, 1 mM EDTA at the pH values indicated.

All buffer chemicals were obtained from Sigma Chemical Co., except for DTT which was obtained from Bio-Rad Laboratories. Potassium phosphite and sodium fluorophosphate were obtained from Alfa. Sodium fluorophosphate was further purified by gradient elution (25 mM/250 mM) with ammonium bicarbonate buffer from a DE52 ion exchange column in the carbonate form at pH 8. This was necessary in order to remove contaminating pyrophosphate, a potent inhibitor of PL-phosphorylase.

RESULTS

Kinetic parameters were determined by means of a Lineweaver-Burke analysis of the initial rate data for each enzyme system at each pH value. The parameters

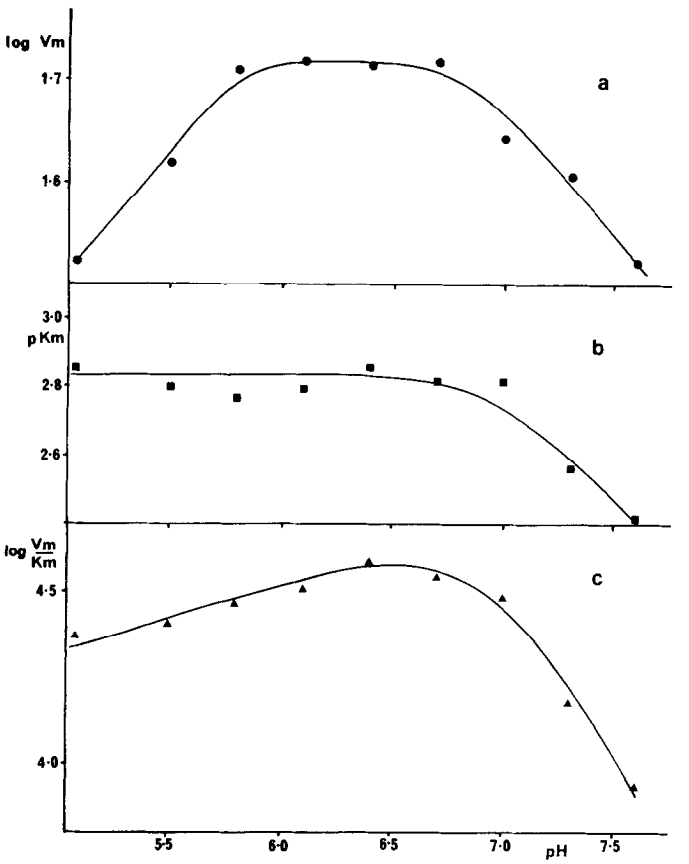

Figure 2. pH dependence of kinetic parameters for phosphorylase b; glucose-1-P as variable substrate in range 2 to 10 mM, in the presence of 1 mM AMP, 1% glycogen.

obtained in this way are plotted (Fig. 1) in the form of log Vm, log Vm/Km and pKm versus pH for both phosphite- and fluorophosphate-activated PL-phosphorylase b. The two profiles obtained for each parameter are very similar, the optimum pH values for the log Vm profiles being within 0.3 pH units of each other. pK_a values for the alkaline limb have been estimated by the unit slope approach (17), and values of 7.0 and 7.3 obtained for the fluorophosphate- and phosphite-activated enzymes respectively. The similarity of these two values is interpreted to mean that the phosphate analogue, and thus the coenzyme phosphate in native phosphorylase, cannot be involved as an essential acid catalyst in the reaction. An involvement as an essential acid catalyst would have required a pK_a difference of approximately 1.8 pH units in such a study. Differences are even less apparent in the profiles shown (Fig. 1b,1c) for pKm and log Vm/Km.

Previous pH profiles for muscle phosphorylase have been determined in the opposite direction, i.e., glycogenolysis. The pH profiles for muscle phosphorylase b in the direction of glycogen synthesis were therefore determined in order to allow a direct comparison of the PL-phosphorylase b data with that of native phosphorylase b. These data, employing gluocose-1-P as the variable substrate, are given in Figure 2, in the form of profiles for the pH variation of the three parameters, log Vm, pKm and log Vm/Km.

CONCLUSIONS

Specific conclusions on the nature of active site groups drawn on the basis of pH-rate profile data are often of dubious validity due to the large number of assumptions necessary to allow interpretation of the data. However, many of these problems have been overcome in this study by means of a direct comparison of pH data obtained from two enzyme systems produced by specific minimal modifications at the active site of one enzyme, namely, the comparison between phosphite- and fluorophosphate-activated PL-phosphorylases. The substitution of a fluorine atom for a proton represents a minimal modification since the Van der Waals radius (1.35Å) of fluorine is not much larger than that of a proton (1.2Å) and considerably smaller than that of the hydroxyl moiety (2.20Å). The major difference arises in the electronic properties of the species and is reflected in their different pK_a values. Thus, the only significant difference between the two phosphorylase analogues being studied is a 1.8 pH unit difference in the pK_a's of an active site group which has been implicated previously as an acid/base catalyst. The absence of any significant differences between the pH/rate profiles for the two species therefore would imply that the coenzyme phosphate is not involved in any essential proton transfer process. Due to the instability of this enzyme system at low pH (<5.5) it was not possible to collect good data on the acid limbs of the pH-rate profiles, but the data on the alkaline limbs are excellent. Therefore it can only be concluded with any degree of certainty that the coenzyme phosphate is not involved as an acid catalyst in the direction of glycogen synthesis. The two sets of data on the acid limbs of the pH profiles, while incomplete, do show certain similarities for the

phosphite- and fluorophosphate-activated systems, implying further that the coenzyme phosphate is not acting as a base catalyst. The 0.3 pH unit difference observed between the apparent pK_a's for the alkaline limbs could easily be the result of slightly different binding characteristics, or enzyme conformations, at the active site in the two cases as a result of the difference in electron negativity.

The argument that the pK_a's of the two phosphate moieties are fortuitously perturbed upon binding such that the two bound species have identical pK_a's and therefore similar activation characteristics is weakened considerably by these latest results. A further indication that there is no differential perturbation of pK_a's arises from a consideration of binding energies. The free energy for such perturbation would most likely be derived from binding free energy, thus would show up in significantly different Km (activation) values for phosphite and fluorophosphate. The measurement of very similar (0.9 and 2.0 mM for phosphite and fluorophosphate respectively) binding parameters therefore argues against such a perturbation.

Direct comparison of these profiles with those obtained for native phosphorylase b (Fig. 2) in the direction of glycogen synthesis raises some interesting similarities and differences. The log Vm profile is very similar, except that for native phosphorylase it was possible to extend the data to slightly lower pH, giving more complete acid limbs. Within the uncertainty inherent in this procedure, the calculated pK for the enzyme-substrate complex on the alkaline side is similar to the 7.2 determined for phosphorolysis (11) as well as to the values for the phosphite- or fluorophosphate activated enzyme determined from Figure 1. The profiles for pKm and log Vm/Km are obviously substantially different. These differences may be due to differences in binding modes, but in any case this does not affect the major conclusion of this paper.

We can therefore conclude from this study that the coenzyme phosphate is not involved as an essential acid catalyst in the enzymic reaction in the direction of glycogen synthesis. A recent paper by Withers et al (3) proposed two possible roles for the coenzyme phosphate based on the proposal that the two

phosphates interact during catalysis. The first of these roles involved the co-enzyme phosphate as an acid catalyst, and the second as an electrophile. We suggest that this data brings strong evidence to bear against the first role, thus would favour the second.

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