STABILIZATION OF A PHOSPHORYLASE *b* ACTIVE CONFORMATION BY HYDROPHOBIC SOLVENTS

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1. Introduction

It is often supposed that glycogen phosphorylase *b* from rabbit skeletal muscle (EC 2.4.1.1) shows an absolute nucleotide requirement for catalytic activity (reviewed [1]). The molecular mechanism by which the enzyme is allosterically activated by nucleotides is still essentially unknown. However, the direct participation of AMP in the catalytic process seems unlikely, since recent crystallographic work has shown that the nucleotide binding site is quite distant from the active site [2,3].

If the action of 5′-AMP simply consists of shifting an allosteric equilibrium from a T to an R state, as assumed in the concerted model for allosteric transitions [4], it is conceivable that structurally unrelated compounds could produce a similar shift, resulting eventually in a bypass of the nucleotide requirement for activity. We have found that, upon addition to the buffer of a variety of hydrophobic compounds, the enzyme activity loses its requirement for AMP. We present here a detailed analysis of this unexpected phenomenon, and we briefly discuss its possible significance with respect to the mechanism of the allosteric activation of phosphorylase.

2. Materials and methods

Crystalline rabbit muscle glycogen phosphorylase *a* and *b* were prepared as in [5]. Limited tryptic digestion of rabbit muscle glycogen phosphorylase *a* was as in [6], and inactivation of muscle glycogen phosphorylase *b* by 2,3 butanedione as in [7]. The AMP site of glycogen phosphorylase *b* was covalently labelled with 8-(*m*-(*m*-fluorosulfonylbenzamido) benzylthio) adenine, kindly given by Dr D. J. Graves [8].

Phosphorylase activity was measured at 24°C in the direction of glycogen synthesis as in [9], care being taken to free all reagents from possible contamination by nucleotides [10,11]. Unless otherwise stated, standard assay medium contained 0.1% oyster glycogen, 10 mM glucose-1-phosphate, and 0.5 mg/ml bovine serum albumin, in 50 mM glycylglycine buffer (pH 6.9), other reagents being added as indicated. In these conditions, phosphorylase *b* is nearly saturated with all substrates when assayed in the presence of saturating AMP (1 mM). This particular activity level is taken as 100% reference. The main conclusions reached here were independently checked by two other assay methods, viz., measurement of the rate of incorporation of [^{14}C]glucose-1-phosphate into a glycogen primer [12], or measurement of the rate of release of glucose-1-phosphate from glycogen and inorganic phosphate [13].

The affinity of the enzyme for activating nucleotides was studied by spectrofluorimetry, taking advantage of the fact that the fluorescence of the AMP analog 1,*N*(6)etheno AMP (ϵAMP) is quenched upon binding to the enzyme [5]. Similarly, the kinetics of ϵAMP binding to the enzyme were investigated using a T-jump apparatus fitted with a fluorimetric detection, as in [14].

3. Results

It has been reported that the addition of 20–30%

(v/v) of some hydrophilic solvents, such as ethylene glycol or dimethylsulfoxide, to the buffer containing phosphorylase *b* had little effect on enzyme activity [8,15]. In contrast, we found that a number of compounds bearing hydrophobic moieties, such as aliphatic alcohols, alkyl-substituted amides, ketones, or ethylacetate cause a marked increase of the very small activity observed in the absence of nucleotide. Preliminary studies showed that among these compounds, aliphatic alcohols were by far the most efficient activators. Figure 1 shows the activity of phosphorylase *b* in the absence of nucleotide as a function of the concentration of several alcohols. Whilst at zero alcohol concentration very little activity is found (0.3% of the activity measured with 1 mM AMP and no alcohol added), this activity is subsequently stimulated and then inhibited when the concentration of alcohol is raised, the maximal activity amounting in some cases to 40% of the reference level. Control experiments showed that the activity decrease is due to a reversible inhibition by high alcohol concentrations and not to irreversible denaturation. Similar experiments run in the presence of 1 mM AMP yielded similar results: upon increasing alcohol concentration, the activity was also first stimulated (up to 150–170% of the reference activity in some cases), and then inhibited. Moreover, alcohol concentrations required to produce maximal activity in the presence or absence of AMP were similar. In contrast, the alcohols did not activate appreciably phosphorylase *a*.

We investigated the influence of the molecular structure of the alcohols on their activation and inhibition properties on phosphorylase *b*. As seen in fig.1, for linear alcohols, the longer the aliphatic chain, the lower the alcohol concentration required to produce both effects. Moreover, for a given number of carbon atoms, linear alcohols were found more efficient than branched ones (fig.1). The same order has been reported for the efficiency with which these alcohols increase the solubility of non-polar amino acids [16], and denature various proteins [16,17]. This suggests that the activation and inhibition processes reflect exposure of non-polar regions of the enzyme to the surrounding medium. It should be pointed out, however, that increasing the alcohol chain length or reducing its degree of branching also favours the inhibition process with respect to the activation process, resulting in a lowering of the maximal activity which can be reached (fig.1). For this reason, we chose *t*-butanol which provides high activation in the absence of AMP (35% of the refer-

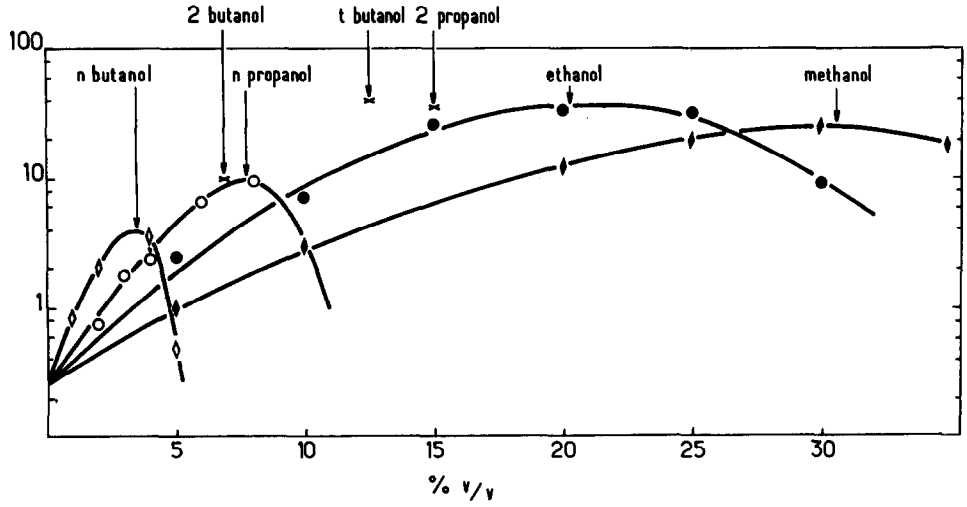

Fig.1. The activity of muscle phosphorylase *b* in the absence of nucleotide, as a function of the concentration of some alcohols. Assay conditions are in section 2. The activity observed in the presence of 1 mM AMP (no alcohol added) corresponds to 100. Only curves referring to activation by linear chain alcohols are shown; crosses denote the position of the maxima observed with branched alcohols.

ence level) for moderate concentrations (12%, v/v, or 1.3 M), as a typical activator for further kinetic studies. Since we were primarily interested in the activating properties of alcohols, only low *t*-butanol concentrations, for which no inhibitory effects are observed, were considered.

In an effort to determine to what extent the AMP-induced and alcohol-induced active enzyme conformations are similar, we undertook a comparison of the kinetic parameters associated with both activations. A distinctive feature of the AMP activation is the existence of a strong positive heterotropic linkage between the activator and the anionic substrates (18). A similar interaction was found when *t*-butanol was used as an activator: with saturating glycogen concentrations, the glucose-1-phosphate concentration required for half maximal activity ($K^{0.5}_{Glc1P}$) decreased from 11–2.3 mM when the alcohol concentration was raised from 5–10% (v/v). On the other hand, in contrast to AMP [19], *t*-butanol showed no positive heterotropic linkage with glycogen. Rather, the K_m for glycogen measured for saturating levels of glucose-1-phosphate was independent of alcohol concentration and much higher than observed in the presence of saturating AMP, suggesting looser glycogen binding.

The activating properties of alcohols observed in the presence of saturating AMP were also further analyzed. As seen in table 1, addition of *t*-butanol (10%, v/v) to the AMP-saturated enzyme affects its kinetic properties in two different ways: (a) the maximal velocity is markedly increased; (b) the glucose-1-phosphate requirement for half maximal activity is greatly reduced. Moreover, the kinetic response with respect to this ligand is non-cooperative, as with phosphorylase *a* (table 1). In fact, all the kinetic properties of phosphorylase *b* in the presence of both AMP and alcohol are strikingly similar to those of the *a* enzyme (table 1), suggesting a similar conformation. This 'a-like' character is also apparent from studies on two physical properties known to differ sharply in the *a* and *b* enzyme, viz., the aggregation state, and the conformation of the AMP site, which can be probed by nucleotide binding kinetics [14]. Thus, in the presence of AMP (1 mM) and

Table 1
Kinetics parameters of the phosphorylase reaction

	Variable ligand	K_m or $K_{0.5}$ (n_{Hill})	V_{max} (μM P_i/min/mg)
Phosphorylase *b* + 5% *t*-butanol	Glc1P	11 mM (1.55)	6
	Glycogen	5×10^{-4}	
Phosphorylase *b* + 10% *t*-butanol	Glc1P	2.3 mM (1.6)	12
	Glycogen	4×10^{-4}	
Phosphorylase *b* + 1 mM AMP	Glc1P	2.4 mM (1.4)	25
	Glycogen	0.5×10^{-4}	
Phosphorylase *b* + 1 mM AMP + 10% *t*-butanol	Glc1P	1.25 mM	35
	Glycogen	$<0.4 \times 10^{-4}$	
Phosphorylase *a*	Glc1P	1.2 mM	25
	Glycogen	0.8×10^{-4}	
Phosphorylase *a* + 0.1 mM AMP	Glc1P	1.0 mM	30
	Glycogen	0.35×10^{-4}	

All glycogen concentrations are given in g/ml (dry wt). The assay conditions are in section 2. The kinetic responses with respect to glycogen or glucose-1-phosphate were measured at 10 mM glucose-1-phosphate and 2.5% glycogen, respectively. Hill coefficients are given in brackets where a cooperative kinetic response was observed

t-butanol (10%, v/v) at 24°C in glycylglycine buffer, phosphorylase *b* sedimented as a tetramer, like phosphorylase *a*, whereas in the presence of any of these effectors alone, it remained essentially dimeric. Turning now to nucleotide binding kinetics, T-jump studies show that, upon progessive addition of *t*-butanol, the enzyme is converted from a conformation characterized by a low affinity for ϵAMP (K_d 100 μM at 11°C) and a short nucleotide residence time (500 μs), to a conformation characterized by a high affinity (10 μM) and a long nucleotide residence time (25 ms). These two latter features have been shown to be characteristic of phosphorylase *a* [5,14].

Two distinct regulatory sites have been identified on the three-dimensional structure of phosphorylase, i.e., the allosteric site on which AMP binds, and the serine 14 residue, which is phosphorylated upon conversion to the active *a* form. In an effort to determine whether interaction of any of these sites with alcohol is responsible for activation, specific experiments designed to modify each of them were undertaken, and the action of alcohols on the modified enzyme was tested. Thus, phosphorylase *b'*, which derives from phosphorylase *a* or *b* through limited tryptic digestion of the N-terminal end of the chain, including serine 14, could be activated by alcohols to nearly the same degree as phosphorylase *b*: this rules out the possibility that the N-terminal end of the chain is the target for alcohol action. Blocking of the AMP site was performed by two specific chemical modifications:

(1) The enzyme was reacted with 8-(*m*-(*m*-fluorosulfonylbenzamido) benzylthio) adenine, which partially activates the enzyme through covalent binding to the AMP site [8]: the modified enzyme, which under our standard conditions showed 10% of the reference activity and could not be further activated by AMP, was still activable 5-fold by *t*-butanol.

(2) Phosphorylase *b* was reacted with 2,3-butanedione, which inactivates the enzyme presumably through reaction with the arginyl residue on which the phosphate group of AMP binds [7]. In our hands, >90% of the activity observed in the presence of 1 mM AMP could be destroyed by this reagent without any loss (<10%) of the *t*-butanol induced activity (fig.2).

Thus, these experiments also rule out the AMP site as the locus for alcohol action. This conclusion is supported by the observation that alcohols and AMP act as synergistic effectors on the enzyme activity.

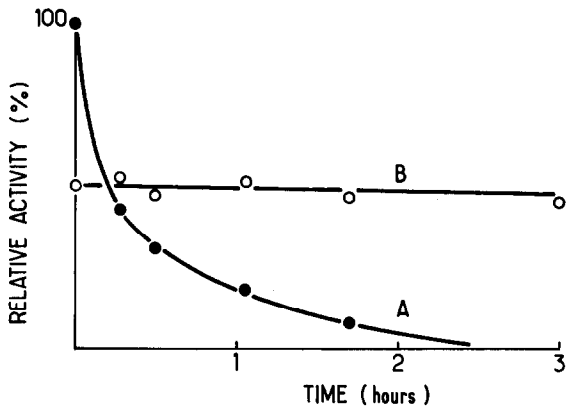

Fig.2. Time course of activity loss after reaction of phosphorylase *b* with 2,3-butanedione. The enzyme (5 mg/ml) was reacted at 30°C with 5 mM 2,3-butanedione as in [7]. After various reaction times, aliquots were diluted into assay medium and tested for activity in the presence of 0.5 mM AMP (A) or 10% *t*-butanol (B).

4. Discussion

To understand the mechanism of the non-specific activation of phosphorylase reported here, it must first be determined whether alcohols activate the enzyme through stabilization of its most active conformations or through lowering the activation energy of the enzymatic reaction. At present, we favour the first interpretation, for the following reasons:

(i) Glycogen phosphorylase *a* behaves as a Michaelian enzyme having the best K_m for substrates and a high turnover number. This very efficient catalyst is not at all affected by alcohols.

(ii) The main action of alcohols on the *b* enzyme in the presence of saturating concentration of AMP is to decrease the K_m for glucose-1-phosphate and to suppress its homotropic cooperativity.

(iii) In the complete absence of AMP strong heterotropic linkage exists between alcohols and glucose-1-phosphate. All these facts suggest that alcohols should be considered as true allosteric effectors and not as co-catalysts of the enzymatic reaction.

What can this work tell about the mechanism of activation of the enzyme? Indeed, it clearly suggests that, during the conversion from inactive to active conformations, some non-polar region of the enzyme becomes exposed to water, a process which should be greatly facilitated by including aliphatic alcohols into the buffer. The free energy gained in this case is apparently sufficient not only to convert all enzyme forms into the most active conformations when both nucleotide and alcohols are present, as indicated by the Michaelian behaviour of the enzyme, but also to stabilize some partially-active conformations even in the absence of nucleotides. Several authors have reported the existence of non polar pockets accessible to solvent on the phosphorylase molecule [20]. Whilst one of them could be the target for alcohol action, we have not been able so far to locate this site by suitable chemical modification experiments, although neither the AMP site nor the N-terminal end of the chain seem involved, nor to estimate the importance of the molecular rearrangement associated with activation. Hopefully, X-ray crystallography will clarify some of these points in the near future.

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