Spin Label Studies on Phosphorylase B

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SUMMARY

Spin label studies upon phosphorylase B have revealed that the temperature-dependent equilibria between different conformational states are strongly influenced by the presence of substrate or allosteric modifier. In the absence of substrate or modifier, the equilibrium involves only two states, while at least three are involved in their presence. Removal of pyridoxal-5'-phosphate produces a conformational change reflected by increased mobility of the label. The apo-enzyme undergoes a structural change in the presence of the allosteric modifier AMP.

Introduction

The enzyme glycogen phosphorylase B, when crystallized from cysteine solution has been shown to contain one highly reactive sulfhydryl group on each monomer unit of molecular weight 92,500 (1), in addition to several other thiol groups of lower reactivity. The reactive sulfhydryl is preferentially substituted by sulfhydryl-specific reagents (1), including iodoacetamide derivatives (2), thereby permitting the introduction of a structural probe at a specific location within the tertiary structure (3). In the present study a spin label (4) placed at this position has been used to monitor local alterations in conformation as a function of conditions.

Experimental

Phosphorylase B was prepared from frozen rabbit muscle (Pel-Freeze, Inc.) by the method of Fischer and Krebs (5). Solutions were freed from AMP by passage

through a 1 X 45 cm Sephadex G-25 column, elating with buffer.

Two nitroxide spin labels, both of which were iodoacetamide derivatives, were purchased from Synvar. The labels were 3-(2-iodoacetamido) -2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (I) and 3-[(2-iodoacetamido) acetamido]- 2,2, 5,5-tetramethyl-1-pyrrolidinyloxyl (II), which differs from I in possessing an additional $-NHCOCH_2-$ group in the linear portion of the molecule.

Conjugation was carried out by adding 500 µl of a 0.2% solution of label in 33% ethanol to 10 ml of a 1% solution of enzyme in 0.1 M glycylglycine, pH 7.0. After 30 minutes at 3°, the conjugated enzyme (0.1 - 0.3 groups per monomer unit) was separated from excess label by passage through a 1 X 25 cm. Sephadex G-25 column, equilibrated with the desired buffer.

A Varian E-40 ESR spectrometer was used for measurements. Typical settings were: field set, 3265 or 3245 G; scan range, 100 or 40 G; time constant, 10 sec.; scan time, 30 min.; microwave power , 17 dB; microwave frequency, 9.185 GHZ; modulation amplitude 1.25 G.

Glass-redistilled water and analytical grade reagents were used for all solutions.

Results

Conjugates of phosphorylase B with spin label I in 0.1 glycylglycine, pH 7.0, 23° showed ESR spectra characteristic of a partially immobilized label (Figure 1) (5). The apparent correlation time, as computed by the method of Stone, et al (6), was 1.4 nsec., which is substantially greater than the value, 0.02 nsec, expected for the free label (6), but considerably less than that predicted for the phosphorylase dimer (∿200 nsec), in harmony with expectations for a label with restricted freedom of rotation.

The ESR spectra show a pronounced temperature dependence, corresponding qualitatively to an increased immobilization of the label with decreasing temperature Using the ratio of peak-to-trough separations for the downfield and center bands as an index of the effect (6, 7), a 20% decrease occurs between 27 and 2° (Table 1). This cannot be attributed solely to the increase in solvent

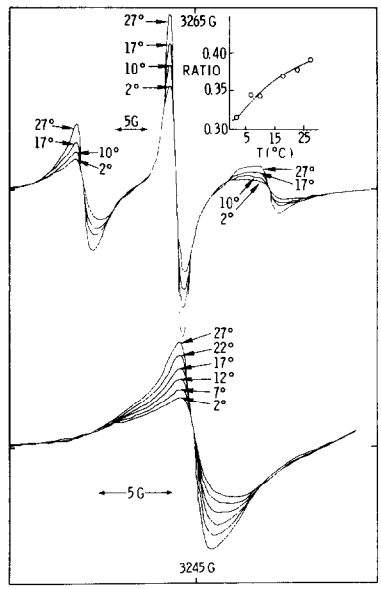

Figure 1: (upper) ESR spectra as a function of temperature for phosphorylase B (3 mg/ml) conjugated with label I in 0.1M glycylglycine, pH 7.0
(inset) Ratio of peak-to-trough separations of downfield and center bands for above conditions as a function of temperature.
(lower) Downfield band as a function of temperature.

viscosity, since the decrease is much less in 30% sucrose, 23°, whose viscosity is greater (4 versus 1.7 centipoise) than that of water at 2° (Table 1). The free label showed only a 2% decrease in ratio between 27° and 2°.

Examination of the spectra at higher resolution reveals the presence of well-defined isosbestic points (Fig 1), suggesting that no more than two different microenvironments are sensed by the label with decreasing temperature. These results are consistent with the proposal (8) that phosphorylase B undergoes a transition to a second conformation at low temperatures and that this conformational change is accompanied by increased immobilization of the spin label.

In the presence of a saturating (10^{-3}M) level of the allosteric activator AMP (9), the ESR spectrum changes, the ratio of the peak-to-trough separations of the downfield and center bands decreasing and the apparent correlation time

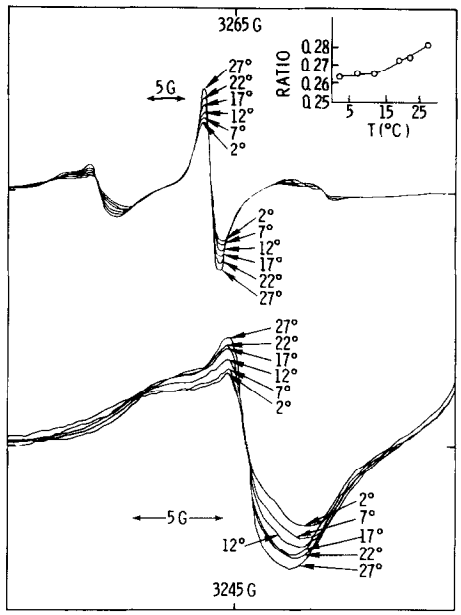

Figure 2: (upper) ESR spectra as a function of temperature for phosphorylase B (3 mg/ml) conjugated with label I in 0.1M glycylglycine, pH 7.0 plus 10^{-3}M AMP.

(inset) Ratio of peak-to-trough separations of downfield and center bands for above conditions as a function of temperature.

(lower) Downfield band as a function of temperature.

increasing (Figure 2 and Table 1). The set of spectra at different temperatures from 27 to 2° do not show an isosbestic point (Figure 2), indicating that the label in this case senses more than two microenvironments, corresponding to transitions between at least three distinguishable conformations in this temperature range.

Phosphorylase B conjugated with spin label II showed at all temperatures ESR spectra indicative of a probe less immobilized than label I (Table 2), with almost no AMP effect at all temperatures. The structural alterations induced by AMP are apparently of sufficiently short range so that an increase in side-chain length by one $-NHCOCH_2-$ unit abolishes the effect upon spin label mobility.

Table 1

Properties of Phosphorylase B Conjugated with Spin Label I under Various Conditions

Medium *	T	Ratio of Downfield to Center Bands
0.1M glycyl-glycine, pH 7.0	23°	0.37
same	2°	0.31
same, plus 30% sucrose	23°	0.34
same, plus 10^{-3}M AMP	22°	0.27
same, plus 0.033M glucose-1-phosphate	22°	0.32
same, plus 0.033M glucose-1-phosphate and 10^{-3}M AMP	22°	0.26
same, plus 1% glycogen	23°	0.36
same, plus 1% glycogen and 10^{-3}M AMP	22°	0.27
same, plus 0.017M phosphate	23°	0.31
0.1M β-glycerol phosphate, pH 7.0	22°	0.295
same, plus 10^{-3}M AMP	22°	0.27
0.4M imidazole-citrate, pH 6.2	23°	0.47
same, plus 0.1M L-cysteine	23°	0.57

* All solutions were 2 x 10^{-3}M in dithiothreitol.

Table 2

Properties of Phosphorylase B Conjugated with Spin Label II in the Presence and Absence of AMP

Medium	T	Ratio of Downfield and Center Bands
0.1M glycyl-glycine, pH 7.0	22°	0.63
	12°	0.58
same, plus 10^{-3}M AMP	22°	0.62
	12°	0.58

Table 3

Properties of Apophosphorylase B Conjugated with Spin Label I in the Presence and Absence of Pyridoxal-5'-phosphate

Medium	T	Ratio of Downfield and Center Bands
0.1M glycyl-glycine, pH 7.0	23°	0.46
same, plus 10^{-4}M PLP	23°	0.36
same, plus 10^{-3}M AMP	23°	0.30
same, plus 0.033M glucose-1-phosphate	23°	0.28

The substrates glucose-1-phosphate and inorganic phosphate both caused a decrease in the downfield to center band ratio for label I (Table 1), the combined effect of glucose-1-phosphate and AMP being greater than that of either effector alone (Table 1) In all three cases, the spectra between 27° and 2° failed to show an isosbestic point, implying a temperature-dependent transition between more than two conformations.

In contrast to the other two substrates, glycogen failed to produce a change in ESR spectra in the presence or absence of AMP (Table 1).

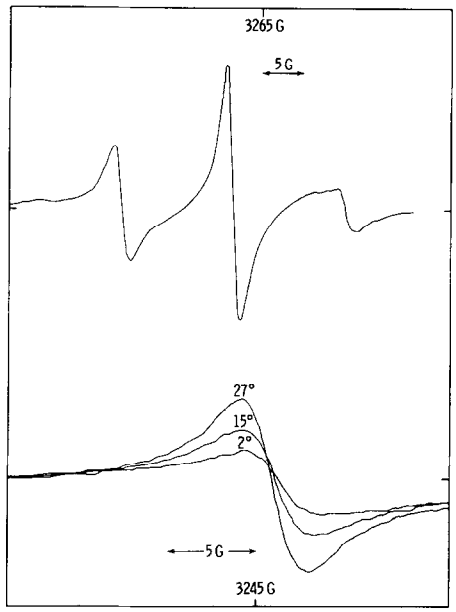

Figure 3: (upper) ESR spectra of apo-phosphorylase B (3 mg/ml) conjugated with label I in 0.1 glycylglycine, pH 7.0 at 23°
(lower) Downfield band of apo-phosphorylase B as a function of temperature.

The ESR spectra were equivalent in 0.1M tris, pH 8.7, and 0.1M glycylglycine, pH 7.0 (Table 1). However, the buffer β-glycerol phosphate, which resembles the substrate, produces a flattening of the downfield band, similar to that produced by AMP or substrate (Table 1).

In the "deforming buffer" 0.4M imidazole-citrate, pH 6.2 (10), the ratio of the downfield to center band heights increases sharply (Table 1). The addition of L-cysteine, which removes the pyridoxal-5'-phosphate (PLP) group causes a further increase in ratio. This increase persists for the apo-enzyme, isolated by the method of Shaltiel, et al. (10) (Table 3). The apparent correlation time in 0.1M glycylglycine, 1.0×10^{-9}, is about 2/3 that of the holoenzyme, indicating a considerably increased mobility of the label (Table 3). The addition of excess (PLP), which reforms the holoenzyme (10) results in a recovery of the ESR spectrum of

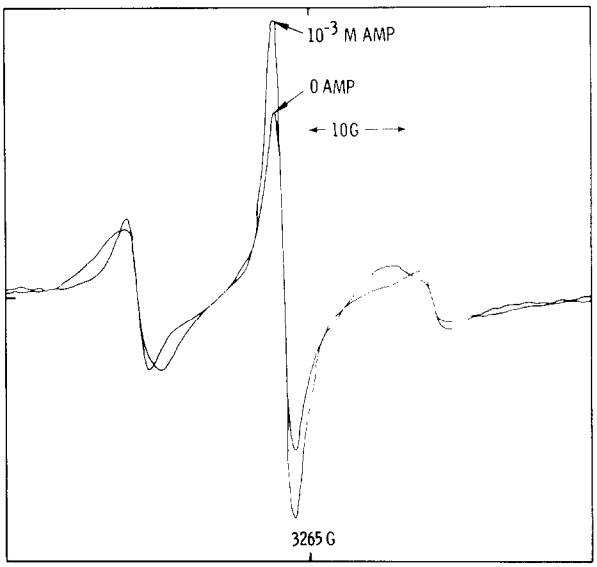

Figure 4: ESR spectra of apo-phosphorylase B conjugated with label I in 0.1M glycylglycine, pH 7.0, 23° in the presence and absence of 10^{-3}M AMP.

the native enzyme (Table 3 and Figure 3). The spectra of the apo-enzyme show a pronounced temperature dependence between 27 and 2° (Figure 4). The absence of an isosbestic point indicates that more than two conformational states must be involved in the equilibria.

The apo-enzyme retains a significant response to AMP and glucose-1-phosphate (Table 3 and Figure 4), the change being in the direction of increased immobilization of the label. Binding sites for AMP and substrate clearly persist for the apo-enzyme, as does their influence on the conformation.

Conclusions

The mobility of a spin label attached to the reactive sulfhydryl of phosphorylase B is clearly a sensitive function of the conformational state of the enzyme. AMP and the substrates glucose-1-phosphate and phosphate (but not glycogen) cause a conformational transition which further immobilizes the label, as does the substrate-analagous buffer glycerol phosphate.

Whereas the label appears to sense a transition between only two conformations with varying temperatures from 27 to 2° in the absence of AMP or substrate, there is indication of a more complicated equilibrium between three or more distinguishable molecular states in their presence.

The removal of PLP results in a local change in conformation which is reflected by an increased mobility of the label. This would be consistent with a "loosening" of the tertiary structure in this region with a concomitant gain of internal degrees of rotational freedom. The binding sites for AMP and substrate appear to remain, despite the loss of enzymic activity under these conditions (10).

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