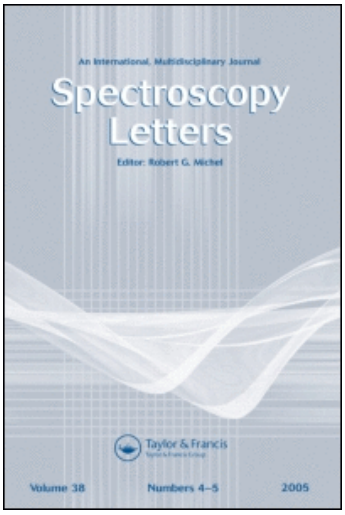

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Spin-Labelling of RUBP Carboxylase-Oxygenase from Puma Rye. I. Properties of the Labelled Enzyme

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SPIN-LABELLING OF RUBP CARBOXYLASE-OXYGENASE FROM PUMA RYE. I. PROPERTIES OF THE LABELLED ENZYME[1]

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This report describes the first application of nitroxide spin labels to the study of structure and function relationships in RUBP carboxylase. RUBPCase (EC 4.1.1.39) is known to be allosterically regulated by several metabolites. Lorimer et al. (1) characterized the activation kinetics of RUBPCase by CO_2 and Mg^{2+} and its inhibition by RUBP. Chu and Bassham (2,3,4) showed that effectors such as NADPH and PGA stimulated the enzyme only when present in the preincubation medium containing HCO_3^- and Mg^{2+}. Buchanan and Schurmann (5) and Vater and Salinkow (6) reported activation of carboxylase activity by fructose-6-phosphate and 6-phosphogluconate.

Evidence for structural changes in RUBPCase upon binding of ligands has been reported. Kwok and Wildman (7) provided evidence of a conformational change in tobacco RUBPCase induced by the binding of RUBP which was recently supported by Grebanier et al. (8).

An HCO_3^- + Mg^{2+} induced conformational change in both hardy (RH) and non-hardy (RNH) forms of rye RUBPCase was reported by Huner and Macdowall (9) and is in agreement with the recent report of Grebanier et al. (8) for the enzyme from pea seedling shoots.

The structural and catalytic properties of rye RUBPCase have been shown to change upon cold-adaptation (9,10,11,12,13). The purpose of this study was to investigate the possible use of the spin-labelling technique to monitor conformational changes in rye RUBPCase with the intention of eventually employing this technique to study the binding of allosterically important ligands. A preliminary account of this work was presented elsewhere (14).

MATERIALS AND METHODS

Rye (Secale cereale L cv Puma) was used throughout this work as the source of the enzyme. Conditions used for germination, and growth at cold-hardening and non-cold-hardening temperatures were described previously (10).

[^{14}C]$NaHCO_3$ was obtained from New England Nuclear, and NEM, RUBP, and DTT from Sigma. SDS was from Pierce Chemical Co. and the spin-label, 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl from Syva, Palo Alto. Sephadex was from Pharmacia.

RUBPCase was purified by gel filtration on Sephadex G-100 and G-200 and finally subjected to ion exchange chromatography on DEAE Sephadex A50 as described by Huner and Macdowall (12).

Both forms of the enzyme were HCO_3^- + Mg^{2+} activated essen-

tially by the method of Lorimer et al. (15) and described in detail elsewhere (9,13).

Spin-labelled RUBPCase was prepared by dissolving the purified enzyme samples (10 to 15 mg) in 0.1 M phosphate buffer (pH 7.4) whereupon the nitroxyl spin-label (20 mg/ml of 95% ethanol) was added in 10 µl aliquots and stirred immediately. After the required ratio of label/protein had been attained, the solution was incubated for 30 min at 23°C. The solution was then centrifuged at 10,000 xg at 4°C for 10 min and the supernatant applied to a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer (pH 7.4) at 23°C. The labelled protein was eluted with the same buffer, collected, pooled and kept on ice until used. The entire labelling procedure was performed in the dark.

All esr spectra were obtained with a Varian E-3 spectrometer with a field scan range of 10 G and a scan time of 16 min. Temperature experiments were performed within the cavity. Protein was routinely determined by the method of Lowry et al. (16) using bovine serum albumin as a standard.

RESULTS

The catalytic activity of the spin-labelled enzyme preparations was first investigated to establish labelling conditions which would allow retention of catalytic activity and allosteric properties. The results of Fig. 1 indicate that RH RUBPCase retained the allosteric property of HCO_3^- + Mg^{2+} activation when

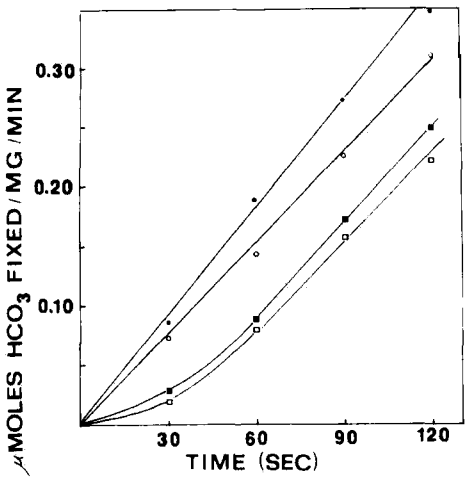

Figure 1. Effect of NEM spin-labelling of RH RUBPCase on enzyme activity and substrate activation. The activity of labelled (open symbols) and unlabelled enzyme (closed symbols) was tested before (squares) and after (circles) HCO_3^- + Mg^{2+} activation as described in Materials and Methods.

label/protein ($\frac{W}{W}$) did not exceed 1/6. however, carboxylase activity was inhibited by about 20% under these conditions. Similar results were obtained for RNH RUBPCase except that label/protein could not exceed 1/12 without excessive loss of carboxylase activity. The enzyme tolerated incubation at low concentrations of unlabelled NEM for up to 2 hr. without significant activity loss. However, the RNH form lost activity at concentrations of NEM greater than $5x10^{-5}$ M while the RH form was partially inactivated above 10^{-4} M.

Fig. 2 illustrates the effect of the ratio of protein to label on the esr spectrum of the NEM label. Spectrum a is a typical 3 line spectrum of a nitroxyl free radical in aqueous

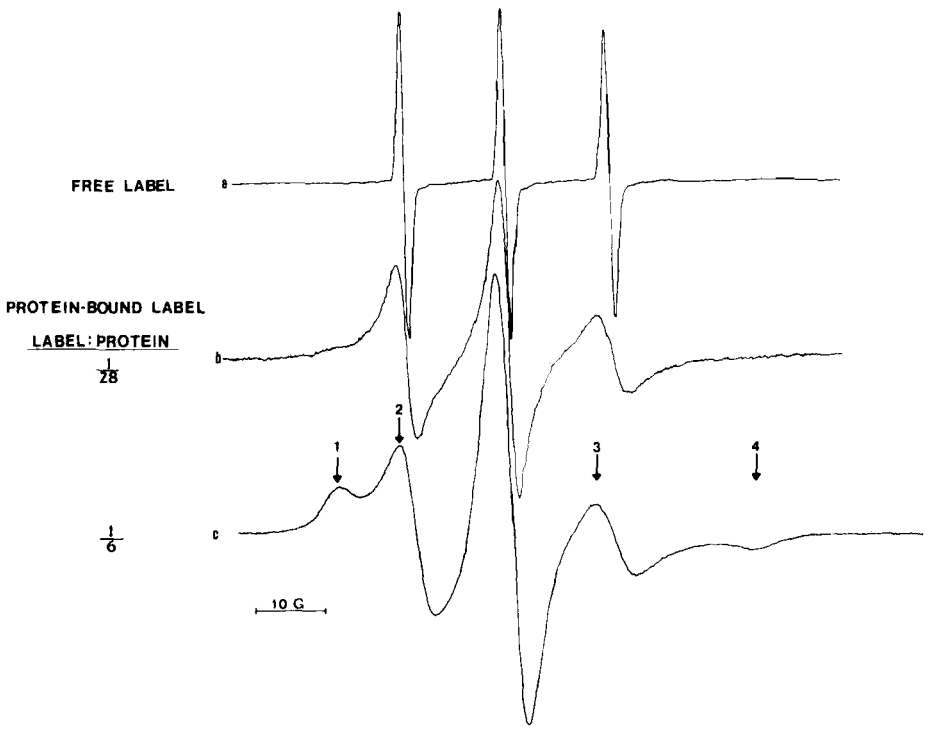

Figure 2. Effect of protein concentration on the spectrum of the NEM spin-label. (a) esr spectrum of the nitroxyl spin-label in 0.1 M phosphate buffer, pH 7.4. (b) esr spectrum obtained when the label/protein was 1/28. (c) esr spectrum obtained when the label/protein was 1/6. RH RUBPCase was used in this experiment. Arrows represent the low field (1 and 2) and high field (3 and 4) components of the spectra a.

solution (17). Upon conjugation of the spin-label to RH RUBPCase (Fig. 3b), there was an increase in the line widths of the three peaks. When the label to protein ratio was increased from 1/28 to 1/6 (Fig. 3c) a more complicated spectrum emerged which contained additional low field (arrow 1) and high field resonances (arrow 4).

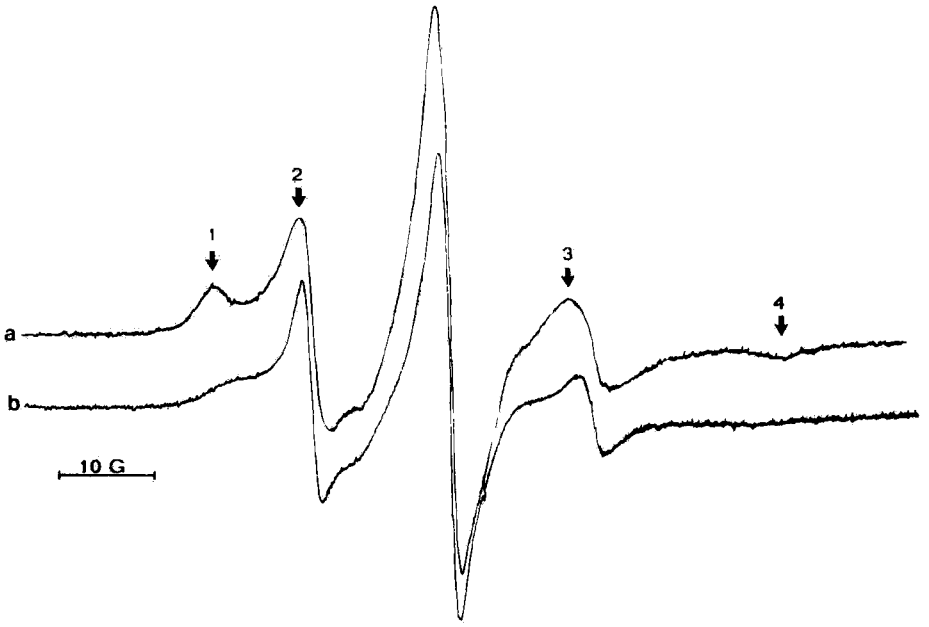

Figure 3. Effect of SDS on the spectrum of spin-labelled RH RUBPCase. The enzyme was labelled such that the label to protein was 1/6 as described in Materials and Methods and then incubated in the absence (a) and presence (b) of 1% SDS at 23°C. Arrows represent low field (1 and 2) and high field (3 and 4) components of the spectra.

To test whether the esr spectrum of the spin-labelled RUBPCase was sensitive to structural changes in the enzyme, the effects of SDS were investigated. The results in Fig. 3 show that both low (arrows 1 and 2) and high field signals (arrows 3 and 4) of the spectrum of spin labelled RH RUBPCase were drastically altered. The intensity of signals 1 and 4 were drastically reduced while the line width of signals 2 and 3 became narrower in the presence of 1% SDS. Furthermore, there was a shift in the low field peak 1

with a concomitant shift in high field peak 3. Fig. 3, spectrum b remained unchanged over a period of several hours at 25°C.

The stability of spin-labelled RH and RNH RUBPCase was investigated at various temperatures. At -23°C, a rigid limit spectrum was obtained with little or no contribution from rapidly tumbling probe molecules. Fig. 4 shows that the effects of freezing on the spectrum of spin-labelled RNH RUBPCase were completely reversible on thawing. Similar results were obtained for RH RUBPCase. Furthermore, the spectra of spin-labelled RH and RNH RUBPCase remained unchanged for at lease 8 hr at 23°C and 24 hr at 4°C under the labelling conditions described in Materials and Methods.

Although the protein-conjugated spin-label was stable in phosphate buffer (pH 7.4), it was not stable at 25°C in the presence of 0.1 M Tris buffer(pH 8.6) and 1mM DTT. Under these conditions, a third component appeared in the esr spectrum of both RH and RNH RUBPCase. The most conspicuous change occurred in the high field signal (Fig. 5 arrow) with the appearance of a new, narrow resonance which increased in intensity with time. As indicated in Fig. 6 (open circles), there was a linear relationship between the height (h^f_{-1}) of this high field signal and the incubation time at 25°C. Furthermore, the presence of HCO_3^- and Mg^{2+} increased the rate of appearance of this new high field peak (Fig. 6, closed circles). After passage of the sample through Sephadex G25, the narrow component disappeared.

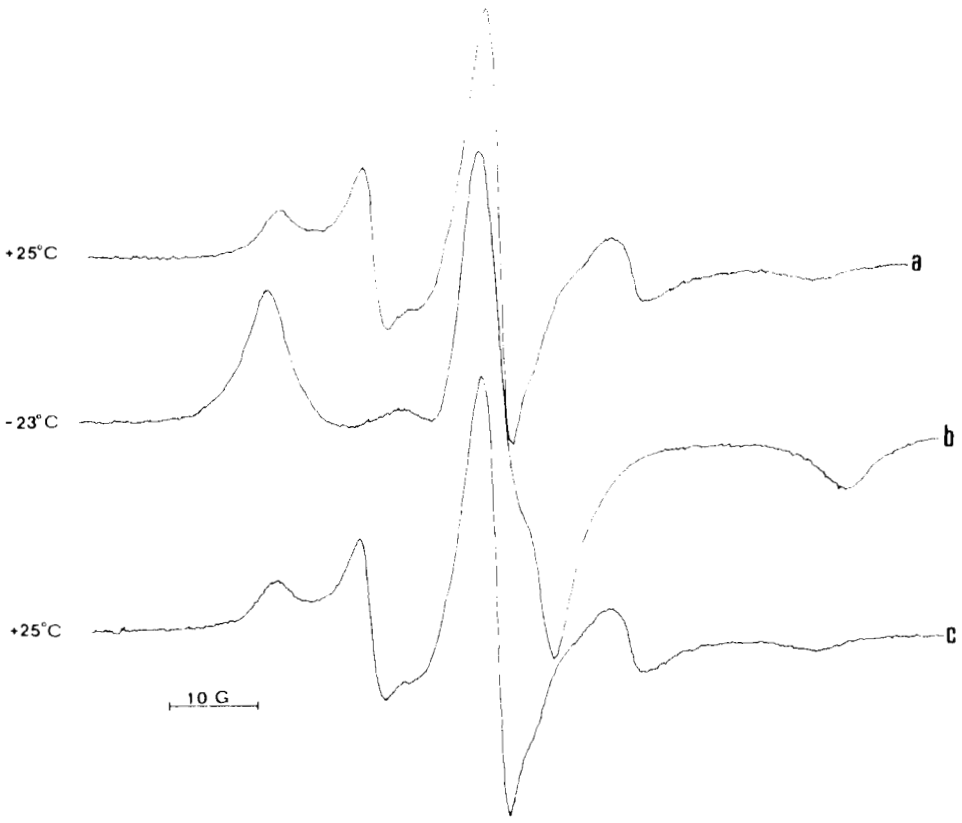

Figure 4. Effect of freezing and thawing on the esr spectrum of spin-labelled RNH RUBPCase. The enzyme was labelled such that label/protein was 1/12 as described in Materials and Methods. (a) spectrum obtained at +25°C, (b) spectrum obtained when same sample was frozen at -25°C. (c) spectrum obtained when the same sample was reheated to + 25°C. The experiment was performed within the esr cavity and the temperature monitored with a thermistor located within the sample tube.

DISCUSSION

Huner and Macdowall (13) reported that free SH groups were associated with the active site of rye RUBPCase. Our results indicated that RH and RNH RUBPCase catalytic activities were

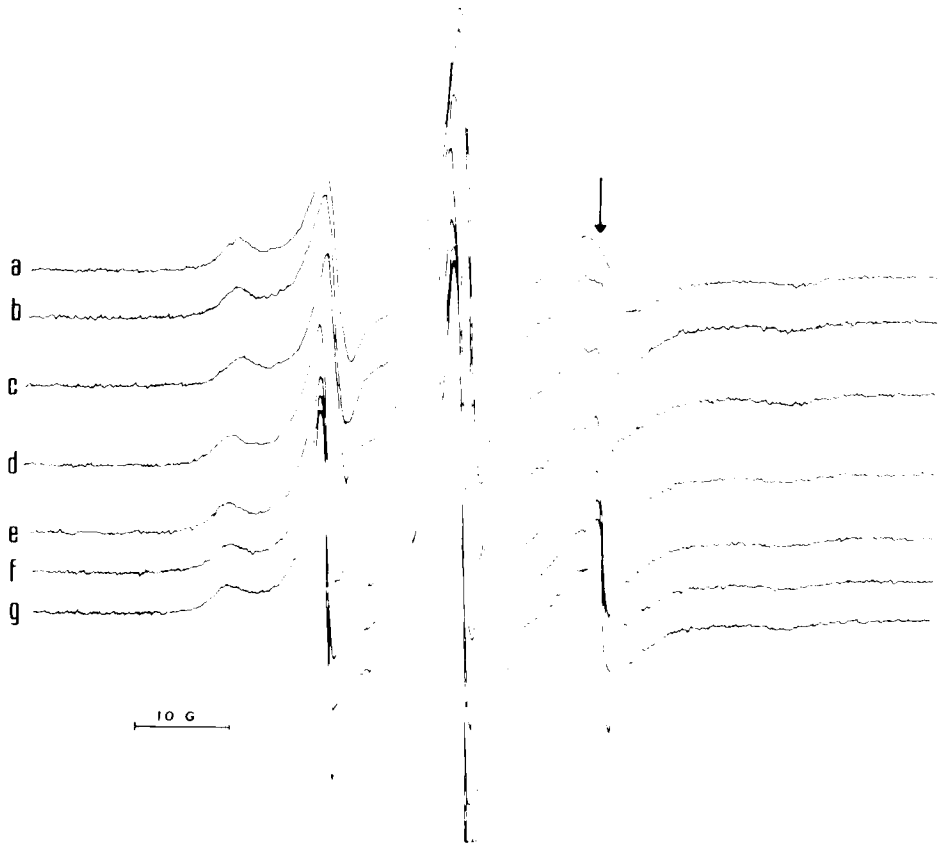

Figure 5. Instability of the spin-labelled RUBPCase at 25°C. RHN RUBPCase was labelled such that the label/protein was 1/12 as described in Materials and Methods. The labelled protein was subjected to gel filtration through Sephadex 25 equilibrated with 0.1 M Tris buffer (pH 8.6) containing 1 mM DTT at 23°C. Labelled protein samples were collected, pooled and immediately put on ice. The spectra were obtained after the following incubation times at 25°C: (a) 0 (b) 15 (c) 23 (d) 45 (e) 65 (f) 120 min. Arrow represents the high field resonance of a third spectral component.

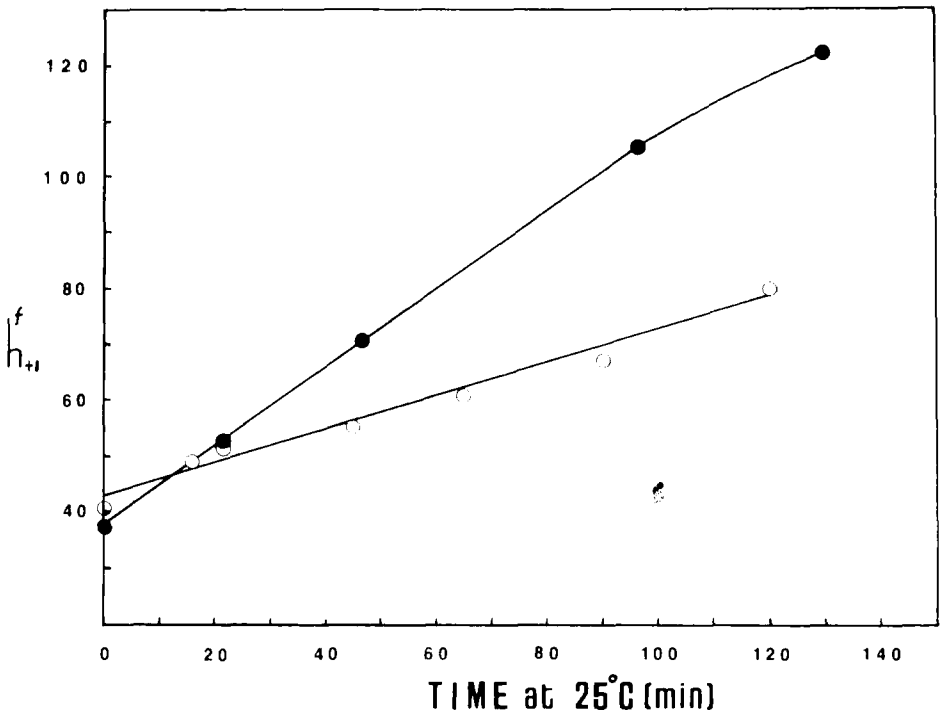

Figure 6. Plot of the increase in peak height (h^f_{+1}) of the high field peak (arrow in Fig. 5) with time. (o): Results obtained from the spectra presented in Fig. 5. Conditions are the same as those described in Fig. 5. (●): Conditions for spin-labelling are identical to those for Fig. 5 except that the labelled enzyme was fully activated in the presence of 0.1 M Tris buffer (pH 8.6) containing 10 mM $NaHCO_3$, 20 mM $MgSO_4$ and 1 mM DTT. Esr spectra were obtained after the following incubation times at 25°C: 0, 23, 45, 100, and 130 min.

sensitive to NEM, above 0.1 mM and 0.05 mM, respectively, a potent SH reagent. This is consistent with earlier reports by Trown et al. (18), Sugiyama et al. (19,20) and Takabe and Akazawa (21) that the activity of RUBPCase from other plant and bacterial sources were inhibited by sulfhydryl reagents.

At concentrations of the NEM spin-label which produced the 2 component spectrum but allowed retention of 80% of carboxylase activity and of allosteric effects of HCO_3^-, spin labelled RH and RNH RUBPCase were virtually identical and esr spectra were composed of two overlapping components; a weakly immobilized spectrum (Fig. 3, scan c, arrows 2 and 3) and a strongly immobilized spectrum (Fig. 2, scan c, arrows 1 and 4). The presence of 2 spectral components is consistent with the work of Cornell and Kaplan (22,23) for NEM spin-labelling of bovine serum albumin and Hidalgo and Thomas (24) who studied spin-labelling of SH groups of proteins in the sarcoplasmic reticulum. Furthermore, the protein concentration-dependence of the two component spectrum is consistent with the previous work of Huner and Macdowall (9) who showed that there were at least two populations of free SH groups present in RH and RNH RUBPCase based on their reactivity to DTNB; one was readily accessible, and the other less accessible to this reagent. The esr spectra presented in this report support the thesis that the more reactive SH groups of rye RUBPCase are in an environment relatively free of steric hindrance as indicated by the weakly immobilized signal, whereas the slowly reacting SH groups are buried and result in the presence of the highly immobilized spectral component. No catalytic activity was lost when the first type of -SH groups were covalently bound to maleimide derivatives. Carboxylase activity was adversely affected when the second category reacted.

The NEM spin-label bound to the enzyme appeared to be unstable under conditions normally employed for HCO_3^- + Mg^{2+} activation (Fig. 5 and 6). Since the additional spectral component disappeared upon gel filtration, we conclude that this new component was due to the presence of free label hydrolyzed from the protein.

The effect of SDS on the spectrum of the NEM spin-labelled enzyme (Fig. 3 b) indicated that this technique is indeed sensitive to the known cstructural changes in rye RUBPCase (9,12). Upon dissociation of rye RUBPCase by SDS, the intensity of the highly immobilized component decreased with a concomitant increase in the intensity of the weakly immobilized spectrum. We interpret this as being due an increase in the rate of tumbling of the spin label bound to previously buried SH groups upon dissociation and unfolding of the native enzyme structure. This is supported by the previous results of SH titration of RH and RNH RUBPCase with DTNB in the presence of SDS (2).

That this change in the spectrum was not due to hydrolysis of the spin-label can be judged by the following criteria: (1) the spectrum observed in the presence of SDS was obtained with the protein in 0.1 M phosphate buffer (pH 7.4), the medium in which the bound spin-label was stable (2) the spectrum obtained in the presence of SDS did not change with time at 25°C (3) a third component corresonding to free spin-label was not observed in the presence of SDS (4) and significant changes in the low field and high field lines of the highly immobilized component of

the spectrum were observed in the presence of SDS in contrast to the spectra obtained under conditions which resulted in hydrolysis of the spin-label.

In conclusion, the results presented in this report indicate that, under proper conditions, spin-labelling of rye RUBPCase may be employed for the study of conformational changes in this important, photsynthetic enzyme.

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FOOTNOTES

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[3]Abbreviations: RUBPCase, ribulose bisphosphate carboxylase-oxygenase; RH, cold-hardened rye; RNH, non-cold-hardened rye; NEM, N-ethylmaleimide; DTNB, 5,5-dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol, SDS, sodium dodecyl sulfate; PGA, phosphoglyceric acid; NADPH, nicotinamide adenine dinucleotide phosphate; esr, electron spin resonance.

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