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AN IMPROVED SPECTROPHOTOMETRIC ASSAY FOR RIBULOSEBISPHOSPHATE CARBOXYLASE

R. McC. LILLEY and D.A. WALKER

Department of Botany, The University of Sheffield, Sheffield S10 2TN (U.K.)

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Summary

The assay for ribulosebisphosphate carboxylase activity (EC 4.1.1.39), by spectrophotometric measurement of NADH oxidation with a coupled enzyme system, is greatly improved by addition of an ATP-regenerating system. There is then close agreement between NADH oxidation and $H^{14}CO_3^-$ fixation. Ribose 5-phosphate can be substituted for ribulose 1,5-bisphosphate for assays on chloroplast extracts.

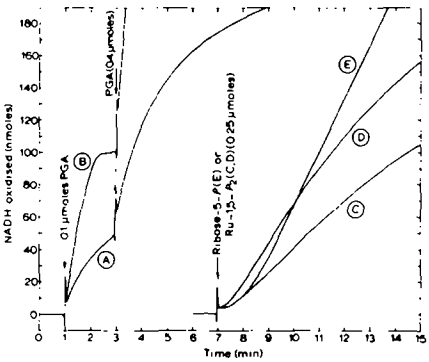
The spectrophotometric assay for ribulosebisphosphate carboxylase (3-phospho-D-glycerate carboxy-lyase (dimerising), EC 4.1.1.39), based on the measurement of 3-phosphoglycerate-dependent NADH oxidation by a coupled enzyme system, is a well-known method [1,2]. This procedure is often more convenient than the radioisotope assay based on $^{14}CO_2$ fixation [3] and gives a continuous measurement of the ribulosebisphosphate carboxylase reaction, but has the disadvantage of a time-lag of up to 1 min between the carboxylation reaction and NADH oxidation [2]. Müller et al. [4] investigated this assay system found that the lag in NADH oxidation resulted from accumulation of 3-phosphoglycerate, and that NADH oxidation was not equivalent to $^{14}CO_2$ fixation on a 2:1 basis. The inadequate rates of 3-phosphoglycerate conversion under the conditions of the ribulosebisphosphate carboxylase assay were attributed to inhibition of the added NAD-dependent glyceraldehyde-3-phosphate dehydrogenase by bicarbonate and also by end-product accumulation effects. We have now found that these effects can be overcome, and a satisfactory assay obtained, by adding an ATP-regenerating system to the reaction mixture.

NADH oxidation in reaction mixtures was measured by extinction at

340 nm using a split-beam recording spectrophotometer with the output adjusted to give a recorder readout corresponding to concentration of NADH (Primary standard: NADH solutions freshly prepared from preweighed vials of desiccated NADH). Reaction mixtures, contained in a 2 mm light-path cuvette, were mixed vigorously for 2 s with an inverted "T" bar after addition of reactant, causing the transient excursions seen on the recorder traces. Chloroplast extracts containing ribulosebisphosphate carboxylase were prepared [5] by osmotic shock of suspensions of intact spinach chloroplasts. The sorbitol included in reaction mixtures was for maintenance of chloroplast integrity in other experiments and was not essential for the assay itself. Solutions of creatine phosphokinase (from rabbit muscle) and mixed 3-phosphoglycerate kinase/NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (from yeast) were prepared daily from freeze-dried solid (all biochemicals were obtained from the Sigma Chemical Co.). Fixation of $^{14}CO_2$ was determined as previously described [6], except that the 10-μl samples, withdrawn from the assay mixture at timed intervals, were immediately injected into 40 μl of 0.3 M HCl, 0.01 M sucrose for determination of acid-stable radioactivity. The total radioactivity in each reaction mixture was determined by injecting 10-μl samples into 0.39 ml of a 0.01 M sucrose solution containing 8% (by vol.) of saturated $Ba(OH)_2$ solution (freshly prepared), which completely fixed $H^{14}CO_3^-$ in the sample. The counting efficiency (determined with [^{14}C]sucrose standards) from both types of sample were identical, when 20-μl aliquots were dried on planchettes and the radioactivity measured in a gas-flow counter. $NaH^{14}CO_3$ was obtained from the Radiochemical Centre, Amersham.

The oxidation of NADH by 3-phosphoglycerate in assay mixtures was examined in the experiments of Fig.1, A and B. Following the addition of 100 nmoles of 3-phosphoglycerate, about 50 nmoles of NADH were oxidised in 2 min in the absence of creatine phosphokinase and phosphocreatine (Trace A). In their presence (Trace B), the reaction was much faster and reached completion in approx. 1 min with stoichiometric oxidation of 100 nmoles of NADH. After the subsequent addition of 400 nmoles of 3-phosphoglycerate, the rate of NADH oxidation in Expt A was markedly non-linear, and the initial rate about a third of that in Expt B. The addition of creatine phosphokinase and phosphocreatine has therefore, by removal of end-product ADP, greatly increased the ability of the reaction mixture to reduce completely 3-phosphoglycerate formed by carboxylation of ribulose 1,5-bisphosphate.

Recorder traces of NADH oxidation with ribulose 1,5-bisphosphate as substrate are shown in Fig.1, C and D. The presence of the ATP-regenerating system (Trace D) shortened the time-lag and considerably increased the rate of NADH oxidation. The decay in rate beyond 4 min was not affected by the use of larger amounts of ribulose 1,5-bisphosphate to start the reaction and may be caused by inactivation of the carboxylase by this substrate [7]. With ribose 5-phosphate as substrate, NADH oxidation in the presence of the ATP-



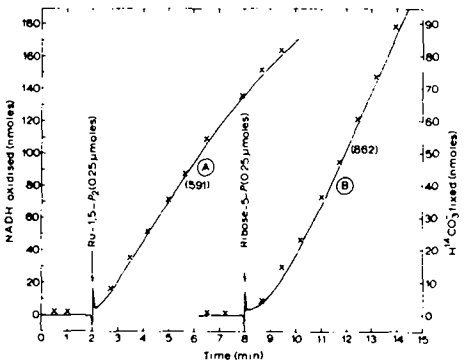


Fig.1. Recorder traces of NADH oxidation. Each reaction mixture contained: 0.33 M sorbitol, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid, 10 mM KCl, 1 mM EDTA, 15 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM ATP, 1 mM NADH, 10 mM $NaHCO_3$, 2.5 units glyceraldehyde-3-phosphate dehydrogenase activity, 3.8 units 3-phosphoglycerate kinase activity and chloroplast extract equivalent to 2.5 μg chlorophyll, pH 7.8, 20 °C, total volume 0.5 ml. In addition, reaction mixtures B, D and E contained 5 mM phosphocreatine and 1 unit creatine phosphokinase activity. After preincubation for 5 min, reactions were started by addition of substrate as indicated PGA, 3-phosphoglycerate; Ru-1,5-P_2, ribulose 1,5-bisphosphate.

Fig.2. Simultaneous measurement of NADH oxidation (recorder trace) and $H^{14}CO_3^-$ fixation (X). Each reaction mixture was identical to those in Fig. 1 including 5 mM phosphocreatine and 1 unit creatine phosphokinase activity but contained 10 mM $NaH^{14}CO_3$ (approx. 30 μCi). The figures in brackets are μmoles NADH oxidised $\cdot mg^{-1}$ chlorophyll$\cdot h^{-1}$, calculated from the rates and the amount of chlorplast extract released per mg chlorophyll by osmotic shock of intact chloroplast preparations. Ru-1,5-*P*, ribulose 1,5-bisphosphate.

regenerating system exhibited a longer initial lag, but the final rate obtained was higher than with ribulose 1,5-bisphosphate and was linear over the time of the assay. The amount of chloroplast extract per assay was chosen to give a maximum rate of NADH oxidation with ribulose 1,5-bisphosphate or ribose 5-phosphate that was no more than 1/3 the rate of NADH oxidation obtained on adding 100 nmoles of 3-phosphoglycerate to the assay mixture.

Simultaneous measurements of NADH oxidation and $H^{14}CO_3^-$ fixation in the reaction cuvette are shown in Fig.2, and compared on the basis of a stoichiometry of two NADH oxidised per $H^{14}CO_3^-$ fixed. With both ribulose 1,5-bisphosphate and ribose 5-phosphate as initial substrates, there was good agreement between the rate of reaction measured by the two methods. The lag between $H^{14}CO_3^-$ fixation and NADH oxidation was 6 to 12 s in both cases. The longer initial lag in the reaction started by addition of ribose 5-phosphate can be attributed to the time required for conversion to ribulose 1,5-bisphosphate. The enzymic activities catalysing this conversion are relatively high in crude chloroplast extracts [8] such as used here. The reason for the higher maximum rates obtained with ribose 5-phosphate (Fig.2) is not known, but may be related to stabilisation effects on the ribulose bisphosphate carboxylase reported by Bahr and Jensen [9]. Inorganic phosphate, present as an impurity, may also contribute to the poorer rate [3] observed with ribulose 1,5-bisphosphate as substrate.

It is concluded that an accurate spectrophotometric assay for ribulose bisphosphate carboxylase can be made when phosphocreatine and creatine phosphokinase are included in the reaction micture. The fact that higher and more linear rates of carbon fixation were obtained with ribose 5-phosphate under these assay conditions shows that this compound may be a preferable substrate to the much more expensive and less stable ribulose 1,5-bisphosphate for routine assays of ribulosebisphosphate carboxylase activity in chloroplast or leaf extracts or any other sample provided adequate activities of ribose-phosphate isomerase (EC 5.3.1.6) and phosphoribulokinase (EC 2.7.1.19) are present.

The results also draw attention to the pitfalls associated with the measurement of ribulose 1,5-bisphosphate carboxylase either by coupled assay in which 3-phosphoglycerate reduction is rate limiting, or by $^{14}CO_2$ fixation in which activity is based on a fixed time interval. Even with the present assay, in which the agreement between the fixation of CO_2 and the reduction of 3-phosphoglycerate is exact (Figs 1 and 2) attention must be drawn to the initial lag with ribose 5-phosphate as substrate and to the lower rate and non-linear kinetics with commercial ribulose 1,5-bisphosphate as substrate. It is clear that some assays have led to erroneous conclusions in the past and that interpretation of kinetics in terms of function should be avoided in the absence of a full understanding of the many variables involved.

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