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NITROGENASE

VI. ACETYLENE REDUCTION ASSAY: DEPENDENCE OF NITROGEN FIXATION ESTIMATES ON COMPONENT RATIO AND ACETYLENE CONCENTRATION

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Summary

Acetylene reduction, an assay for nitrogenase activity (nitrogen:(acceptor) oxidoreductase, EC 1.7.99.2), is dependent on the ratio of the two protein components of nitrogenase as well as on C_2H_2 concentration. As the component I : component II ratio (based on activity) is increased, the C_2H_2 reduction : N_2 fixation ratio decreases to a minimum of 3.4 and then increases. The minimum is found at a ratio near 1 : 1. At a component I : component II ratio of 20 : 1, the C_2H_2 reduction : N_2 fixation ratio is 5.3. Acetylene exhibits substrate inhibition in assays for nitrogenase activity. Both the apparent K_m and K_i for acetylene vary as a function of the relative concentration of components I and II present in the assay. When the more labile component II is limiting in the assay and "saturating" levels of C_2H_2 (above 0.1 atm) are used, N_2-fixation capacity may be greatly under-estimated.

Introduction

Reduction of N_2 to ammonia is the natural reaction catalyzed by nitrogenase (nitrogen:(acceptor) oxidoreductase, EC 1.7.99.2). Assays of this reaction have several limitations. First, N_2 is relatively insoluble, so that 1 atm N_2 gas phase yields levels of N_2 in aqueous phase only about 10 times above the apparent K_m in vitro [1]. Observed rates of N_2 fixation may thus depend on the rate of transport of N_2 across the gas-liquid boundary, making volume to surface area and shaking rate critical variables in the assay. Further, NH_4^+ detec-

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tion with Nessler's reagent is not sensitive enough to determine very low levels of nitrogenase activities reliably.

Among many alternate substrates [2—8], acetylene is more frequently used as a substrate for assays of nitrogenase activity [9—16] because of the sensitivity and convenience of the assay. High levels of acetylene in the assay are found to be inhibitory [10]. As shown in this paper, the inhibitory effect of acetylene is more pronounced when component II (Fe-protein), the electron-donating component [17], is limiting in the assay.

Materials and Methods

A. vinelandii OP was used throughout these studies. Growth of the organism, preparation of extracts and assay conditions for N_2 fixation and acetylene reduction were as described by Shah et al. [18]. Component I and component II were purified by the method of Shah and Brill [19] and component activities were determined by titrating with complementary component [18]. Protein was determined by the biuret method of Gornall et al. [20] with bovine serum albumin as a standard.

ATP, creatine phosphokinase (ATP : creatine phosphotransferase, EC 2.7.3.2), deoxyribonuclease I (deoxyribonucleate oligonucleotidohydrolase, EC 3.1.4.5), and Tris base were obtained from Sigma Chemical Co., St. Louis, Mo. Creatine phosphate was obtained from Pierce Chemical Co., Rockford, Ill. Nessler's reagent was obtained from Paragon C and C Co., New York, N.Y. All other chemicals and gases were of analytical grade available commercially.

Results

Table I shows the results of a typical experiment in which the ratio of component I : II was varied, keeping component II constant for all the assays. The data reveal that the highest C_2H_2 reduction and nitrogen fixation [21] activity is attained with 2—4 times more component I than is needed for highest specific activity. The important point to be observed in these data is that there is a rather sharp minimum in the C_2H_2 : N_2 reduction ratio. This occurs where specific activity is highest, not where total activity is greatest. Similar results were obtained in other experiments using components of varying degrees of purity.

The relative ratios of C_2H_2 : N_2 reduction also vary with the extent to which the reducible substrates are able to saturate the active enzyme complex. Fig. 1 shows the extent of substrate reduction with different levels of C_2H_2 as a function of component ratio. The apparent ratios of C_2H_2 : N_2 reduction are also shown. With a large excess of component I present in the assay, both the N_2- and C_2H_2-reducing activity fall. The decrease is 4-fold for N_2 reduction, and 2.5- and 5-fold for the low and high level of C_2H_2 respectively. In fact, at the high ratio of component I : II, there is more C_2H_2 reduced at the low substrate level than at the high one.

To insure that the effects we have observed are not spurious results caused by limitations of the assay system, we tested two different ratios of components with two levels of C_2H_2, taking samples at several different times. The

TABLE I

EFFECT OF COMPONENT RATIO ON THE RATE OF ACETYLENE REDUCTION AND NITROGEN FIXATION

Ratio* of component I : component II	Acetylene reduction		Nitrogen fixation		Ratio of acetylene : nitrogen reduction
	Per assay (nmol/min)	Relative specific activity**	Per assay (nmol/min)	Relative specific activity**	
0.13 : 1	43.3	38.1	11.0	9.7	3.94
0.33 : 1	98.7	65.6	25.7	17.2	3.82
0.67 : 1	173.1	102.7	48.0	28.8	3.57
1.33 : 1	267.6	115.5	78.7	33.8	3.42
2.67 : 1	350.2	94.8	90.7	24.7	3.84
5.3 : 1	352.2	55.9	87.3	13.8	4.05
10 : 1	266.6	24.4	64.3	5.8	4.21
20 : 1	125.3	5.9	24.0	1.1	5.3

* Amounts of component activity (expressed as nmol of substrate reduced per min) present in the assay were determined directly from the titration curve, using the activity per aliquot of component I added to excess component II (initial points) for activity of component I, and the maximum activity observed as that for component II. The specific activities were over 1600 nmol/min per mg protein for component I and 1060 nmol/min per mg protein for component II. 330 μg component II was used per assay. Component I was a crystalline preparation dissolved in 0.01 M Tris · HCl buffer, pH 7.4, and component II was purified by preparative electrophoresis [19]. Ratio of components is that of activity units present in assay. Assays were incubated at 30°C for 15 min in water-bath shaker.

** Relative specific activity equals nmol acetylene or nitrogen reduced per min per assay divided by the sum of units of component activity present in the assay.

rate of ATP hydrolysis per pair of electrons transferred increases with increasing concentration of component I in the assay [22] and could result in either exhaustion or some inhibition of the creatine kinase-coupling system during a set-time assay. That this is not the case, is clearly seen from Fig. 2. We also tested the effect of increasing and decreasing the creatine kinase levels 4-fold over the concentration used in conventional assay, and obtained identical results. We also used a standard assay stopped by adding trichloroacetic acid at various times and obtained the same results. All of the unstopped assays show a slight lag when begun by adding the enzyme to the assay mixture equilibrated with C_2H_2 at 30°C. We have found a similar lag when assays are begun with addition of C_2H_2. There is usually no such lag in dithionite oxidation assays [18,23], so the effect is probably primarily in the diffusion of C_2H_4 out of the aqueous phase.

The assays shown in Fig. 2 were allowed to go to completion to obtain a measure of the substrate reduced for a fixed amount of ATP hydrolyzed. Table II shows the effect of variation in component ratio and C_2H_2 concentration on both the rate of substrate reduction and the final extent of the reaction. These are only relative measures since at low C_2H_2 concentrations, the substrate was 80—90% reduced before ATP was depleted. The important points to be noted in this table are that the lower ratio of components is considerably more efficient in overall yield at both levels of C_2H_2 and that the relative rates of C_2H_2 reduction at high C_2H_2 concentration decreases with an increasing component I : II ratio while at low C_2H_2 concentration the relative rate of C_2H_2

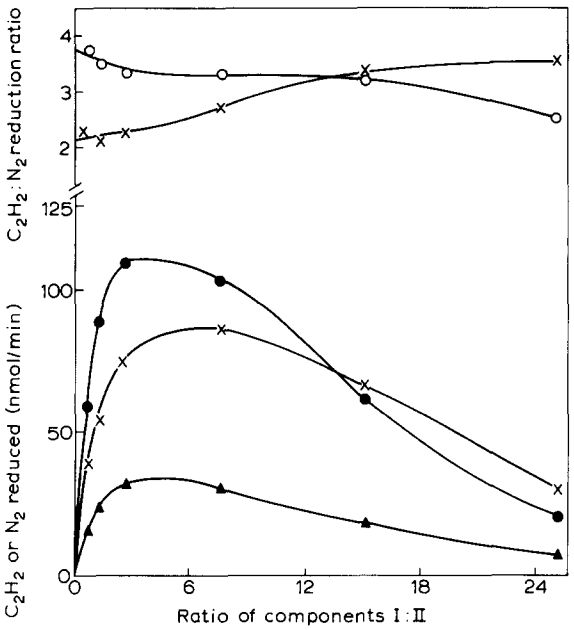

Fig. 1. Relative reduction of acetylene and nitrogen as a function of component ratios and substrate concentration. Assays were carried out in standard assay systems described by Shah et al. [18]. Relative specific activities and component ratios were derived as indicated in the legend of Table I. Component I was a crystalline preparation dissolved in 0.25 M NaCl in 0.025 M Tris · HCl buffer, pH 7.4 [19]. Component II was in 0.35 M NaCl in 0.025 M Tris · HCl buffer, pH 7.4, and was a purified preparation from the second DEAE-cellulose column. All assays were brought to constant ionic strength. Salt-corrected [18] specific activities were 1125 nmol/min per mg protein of component I and 250 nmol/min per mg protein of component II. ●——●, activities with 0.125 atm C_2H_2; X——X, activities with 0.0125 atm C_2H_2; ▲——▲, activities with 1 atm N_2; ○——○, ratio of activities at 0.125 atm C_2H_2 over 1 atm N_2; X——X, ratio of activities at 0.0125 atm C_2H_2 over 1 atm N_2.

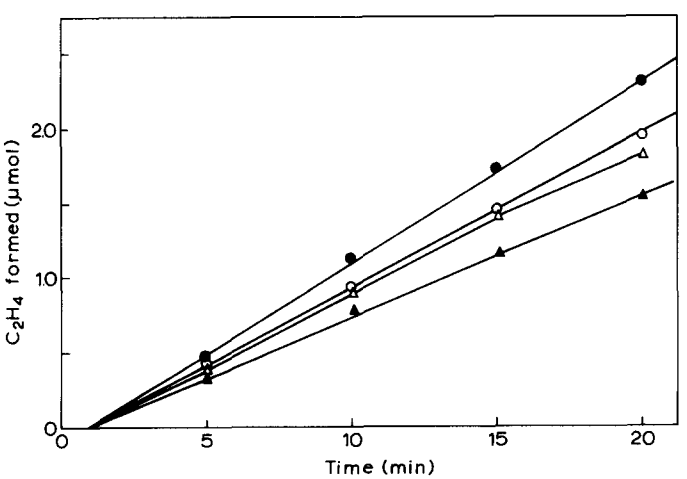

Fig. 2. Rates of acetylene reduction as a function of substrate concentration and component ratios. Assays were started by addition of 0.2 ml of nitrogenase components mixture consisting of either 250 units of component I plus 178 units of component II (component ratio 1.4 : 1) or 2500 units of component I and 178 units of component II (component ratio 14 : 1). Component ratio is defined as in legend of Table I. Aliquots (0.3 ml) of the gas phase were taken at 5-min intervals, after adding an equivalent amount of He to the assay vials. Specific activity of component II was 800 nmol/min per mg protein, 225 μg of component II was used per assay. Specific activity of component I was 1600 nmol/min per mg protein. Component I was a crystalline preparation dissolved in 0.25 M NaCl in 0.025 M Tris · HCl buffer, pH 7.4. Component II was in 0.35 M NaCl in 0.025 M Tris · HCl buffer, pH 7.4, and was a purified preparation from the second DEAE-cellulose column [19]. ●——●, activities with 0.080 atm acetylene and 1.4 : 1 component ratio; ○——○, activities with 0.080 atm acetylene and 14 : 1 component ratio; ▲——▲, activities with 0.0080 atm acetylene and 1.4 : 1 component ratio; △——△, activities with 0.0080 atm acetylene and 14 : 1 component ratio.

TABLE II

EFFECT OF COMPONENT RATIO AND ACETYLENE CONCENTRATION ON APPARENT EFFICIENCY OF SUBSTRATE REDUCTION

See Fig. 2 for details of the assay and determination of component activities. All rates were linear for at least 20 min. Final yield was determined after 1.5—2 h incubation.

Ratio* of component I : component II	C_2H_4 formed using 0.076 atm C_2H_2		C_2H_4 formed using 0.0076 atm C_2H_2	
	Rate (nmol/min)	Yield (nmol/assay)	Rate (nmol/min)	Yield (nmol/assay)
1.4 : 1	130	7600	86	4450
14 : 1	108	5170	105	3900

* Defined as in legend of Table I.

reduction increases with increasing component I : II ratio. As an example of the potentially erroneous results obtained using acetylene concentrations in excess of 0.1 atm and extracts of unknown component ratio, we show Fig. 3. The component II level in all of the assays was constant as the same size aliquot of freshly prepared extract was used in all the assays. Component I levels were varied by supplementing extract with purified component I, thus creating a situation of limiting component II in the extract. A striking dissimilarity in saturation curves for C_2H_2 is noticed. The data in Fig. 3 reveal that observed

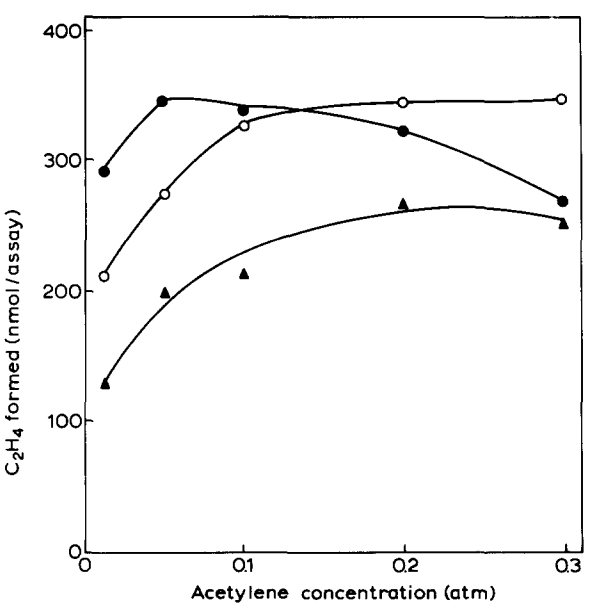

Fig. 3. Effect of component ratio on acetylene reduction as a function of substrate concentration. Component ratio is defined and determined as in legend of Table I. Assays were incubated at 30° C for 15 min in a water-bath shaker. The source of component II was a crude extract [18] with a final protein concentration in the assay of 1.2 mg/ml. ▲———▲, activities with 1 : 1 component ratio; ○———○, activities with 3 : 1 component ratio; ●———●, activities with 11 : 1 component ratio.

activity increases with increasing acetylene concentration until 0.2 atm when component I : component II levels are optimal (approx. 1 : 1). A small excess of component I (component I : II ratio of approx. 3 : 1) overcomes the dilution effect [18] and 0.1—0.2 atm of C_2H_2 "saturates" the system. In contrast, if an excess of component I (components I : II ratio of approx. 11 : 1) is present, the system is "saturated" at 0.05 atm of C_2H_2 and the observed activity drops rapidly with increase in partial pressure of C_2H_2 in excess of 0.1 atm. Similar inhibitory effects of acetylene concentration are observed when activities of carefully prepared fresh extracts are compared with extracts in which component II is partially denatured during handling or storage.

Discussion

It is quite apparent from the data in Figs 1 and 3 that the saturation curve for C_2H_2 will depend on the relative amounts of the components present in an assay. Similarly the estimated N_2-reducing capacity of a system will vary with the component ratio at a fixed C_2H_2 level. There are several implications of these findings. Because of its extreme lability, component II may be limiting in extracts [24—26]. Assays of C_2H_2 reduction by these extracts may greatly underestimate the true N_2-fixing capacity of these extracts if the usual 0.1—0.3 atm C_2H_2 is used in the assays. In *Klebsiella pneumoniae* by contrast, it is component I which becomes limiting during in vivo O_2 inactivation of nitrogenase [27]. To estimate the true N_2-fixing capacity from an acetylene reduction assay, a saturation curve for acetylene should be derived for each new preparation being studied.

These results also indicate that there are great pitfalls in doing kinetic studies of nitrogenase when the ratio of components in an extract is unknown. The apparent K_m for C_2H_2 is much lower when there is an excess of component I than when component I is limiting in the assay (Table II and unpublished data). When component II is limiting, the steady-state concentration of the oxidized form of component I is greater [17] whereas when component I is limiting, reoxidation of component I by substrate is probably the rate-limiting step of the overall reaction.

If other reducible substrates show similar behavior to that of C_2H_2, the degree of substrate inhibition may vary with the ratio of components. Efficiency of reduction to alternate products (e.g. methylamine or methane and ammonia from cyanide) may be critically dependent on the ratio of components present.

Titration of one component against the other until optimum specific activity based on both components is reached [18,21] will provide a suitable system for extensive studies of kinetic parameters. The components need not be pure if a complete titration is carried out and one of the two components can be obtained free of the other, since the optimum point of specific activity is a simple incremental function. It should be clear, at least, that precise conditions under which results are obtained must be clearly specified, including the extent to which one or the other components is limiting in the assay.

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