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Interactions of heterologous nitrogenase components that generate catalytically inactive complexes

(inhibition of nitrogenase/binding of nitrogenase components/binding ratios)

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ABSTRACT **A unique method is described for inhibiting nitrogenase. When *Clostridium pasteurianum* nitrogenase is assayed in the presence of the Mo–Fe protein of *Azotobacter vinelandii*, all the characteristic activities of nitrogenase are inhibited. *C. pasteurianum* nitrogenase is unaffected by the Fe protein of *A. vinelandii*. The Fe protein, but not the Mo–Fe protein of *C. pasteurianum*, inhibits *A. vinelandii* nitrogenase. Both inhibitions described result from the formation of an inactive complex of *A. vinelandii* Mo–Fe protein and *C. pasteurianum* Fe protein. Complex formation requires active components, as oxygen-denatured proteins are ineffective. The results for titration of components of the complex against each other and kinetic data each indicate that the inactive complex consists of two molecules of *C. pasteurianum* Fe protein per molecule of *A. vinelandii* Mo–Fe protein. The results of kinetic experiments suggest that the Fe protein from each organism competes for the same site(s) on the *A. vinelandii* Mo–Fe protein. The Fe protein of *C. pasteurianum* will form an active or an inactive complex with the Mo–Fe proteins from six different organisms. Inhibition by nitrogenase components that form inactive complexes provides numerous ways to study the mechanism of nitrogenase action.**

Nitrogenase from a variety of organisms has been separated into two proteins; one containing molybdenum, non-heme iron, and acid-labile sulfur (Mo–Fe protein) and the other, non-heme iron and acid-labile sulfur (Fe protein) (1). Neither protein alone displays any of the activities characteristic of nitrogenase, but together they are catalytically active when supplemented with a suitable reductant ($Na_2S_2O_4$ furnished *in vitro*) and MgATP. There is limited experimental evidence that the two proteins must bind together to become catalytically effective. Eady (2) found that a 1:1 molar ratio of *Klebsiella pneumoniae* components produced a single sedimentation-velocity peak that corresponded to a sedimentation coefficient considerably greater than that of either protein alone. Higher ratios of Kp2:Kp1* produced an additional peak corresponding to free Kp2. Sedimentation-velocity analyses of *Azotobacter chroococcum* nitrogenase proteins also indicated a 1:1 molar ratio (3). Thorneley (4) obtained evidence of tight complex formation from stopped-flow kinetic experiments. The dissociation constant for *K. pneumoniae* nitrogenase was calculated to be <0.5 μM with a complex consisting of 1 Kp1:3 Kp2. Others have suggested that the catalytically active complex contains 2 Fe protein:1 Mo–Fe protein (5–8). M.-Y. Tso and L. Davis (unpublished observations) noted that additions of *Azotobacter vinelandii* Mo–Fe protein (Av1)* to *Clostridium pasteurianum* nitrogenase (Cp1 + Cp2) inhibited dithionite oxidation; high levels of Av1 completely abolished dithionite oxidation. Their results suggested that Av1 and Cp2 formed an abortive complex, and in this report we describe some properties of this complex.

* The nomenclature of Thorneley and Eady (9) is used. The nitrogenase components of various organisms are designated by a capital letter indicating the genus, a lower case letter indicating the species, and the number 1 or 2 indicating the Mo–Fe protein or Fe protein, respectively. Kp = *Klebsiella pneumoniae*, Cp = *Clostridium pasteurianum*, Av = *Azotobacter vinelandii*.

MATERIALS AND METHODS

The mixture for measurements of nitrogenase activity contained in 1 ml: 10 μmol of $MgCl_2$, 5 μmol of ATP (Sigma Chemical Co.), 40 μmol of creatine phosphate (Pierce Chemical Co.), 14.5 units of creatine kinase (EC 2.7.3.2.; Sigma Chemical Co., 145 units/mg of protein), 200 μmol of $Na_2S_2O_4$ (J. T. Baker Co.), 40 μmol of 2(*N*-morpholino)ethanesulfonic acid (Mes) buffer (Sigma Chemical Co) at pH 6.6, and nitrogenase proteins as indicated. The assay components minus $Na_2S_2O_4$ and nitrogenase proteins were added to a 22 ml vaccine bottle fitted with a vaccine stopper; it was evacuated and refilled three times through a hypodermic needle with cylinder gases (Matheson Gas Products) which had been passed over BASF catalyst R3-11 (Chemical Dynamics Corp.) at 130° to remove O_2. Bottles in which ammonia formation was measured contained 1.0 atmosphere of N_2, and those for measuring acetylene reduction contained 0.1 atmosphere of C_2H_2 and 0.9 atmosphere of H_2. Acetylene was generated by adding calcium carbide to water. An anaerobically prepared solution of $Na_2S_2O_4$ was added to the assay bottles; the bottles were stored on ice until needed, whereupon they were warmed to 30° in a shaking-water bath and the enzyme was injected with a gas-tight syringe to start the reaction. Unless otherwise stated, the two interacting proteins (Av1, Cp2) were preincubated about 1 min before the assay was initiated by the addition of the third protein. Samples for ethylene production were withdrawn from the reaction bottles at indicated times in 1 ml plastic syringes fitted with 27-gauge needles. Assays were terminated by adding 1 ml of a saturated K_2CO_3 solution. Ethylene was analyzed on a Varian 600D gas chromatograph equipped with a flame ionization detector and a column of Porapak R 150 cm long and 2 mm internal diameter; the column was operated with 0.5 ml gas samples at 50° with N_2 as carrier gas. Ammonia formed was determined by the method of Chaykin (10) after microdiffusion (11).

The nitrogenase proteins from *C. pasteurianum* were purified by the method of Tso *et al.* (5). *A. vinelandii* component proteins were purified by a modification of the method of Shah and Brill (12). Protein determinations were performed by the microbiuret (13) or the Lowry *et al.* (14) method with bovine serum albumin (crystallized, Sigma Chemical Co.) as the standard. The micromolar equivalent concentration of each component has been calculated by assuming the molecular weights of Av1, Av2, Cp1, and Cp2 are 216,000 (15), 64,000 (15), 210,000 (16), and 56,000 (16), respectively, and that the

Table 1. Inhibition of *Clostridium pasteurianum* nitrogenase by *Azotobacter vinelandii* Mo–Fe protein

Micromolar equivalents				Total nmol of C_2H_4 formed		
Cp1*	Cp2*	Av1*	Av2*	5 min	10 min	15 min
0.2	2.20			236	496	751
0.2	2.20	0.24		159	332	500
0.2	2.20	0.48		99	216	333
0.2	2.20	0.60		55	123	195
0.2	2.20	0.72		10	27	52
0.2	2.20	0.96		0	0	0
0.2	2.20	1.92		0	0	0
0.2	2.20	0.72†		206	399	614
0.2	2.20		0.62	218	499	716
0.2	2.20		1.25	201	440	648
0.2	2.20		3.12	193	419	615
0.2	2.20		3.12†	215	449	676
0.2			3.12	0	0	0
	2.20	0.24		0	0	0

* See footnote on nomenclature.
† Proteins were inactivated with O_2 by exposure to air at 0°. The Av1 still retained 10% of its original activity after the exposure to air. Cp2 was completely inactivated by the treatment.

maximum specific activity of each protein is 3000 nmol of C_2H_4/(min × mg of protein).

RESULTS

A. vinelandii Mo–Fe protein (Av1) inhibits acetylene reduction by *C. pasteurianum* nitrogenase (Table 1). We conclude that the inhibition is specific for Av1 because Av2 or bovine serum albumin (data not shown) has no effect. In comparison, *C. pasteurianum* Fe protein (Cp2) inhibits N_2 reduction by *A. vinelandii* nitrogenase (Table 2), but Cp1 does not. Oxidation of $Na_2S_2O_4$ and ATP hydrolysis also are inhibited. Inhibition of nitrogenase activity occurs only when Av1 and Cp2 are two of the three nitrogenase components present in the reaction mixture. The heterologous crosses of Av1 + Cp2 or Cp1 + Av2 catalyze no detectable acetylene reduction, N_2 reduction, $Na_2S_2O_4$ oxidation, or ATP hydrolysis above background levels. The inhibition depends upon active protein, as inactivation of the inhibitory components with O_2 returns their inhibitory capabilities to levels comparable to those of the noninhibitory components (Tables 1 and 2). An aliquot of essentially pure Cp2 was incubated anaerobically in the presence of 0.5 mM $Na_2S_2O_4$ for 1 hr at 0°; this cold-inactivated protein produced 13.5% inhibition of acetylene reduction by *A. vinelandii* nitrogenase compared to 70.6% inhibition when the Cp2 was maintained at 10–15°, a temperature that does not induce cold inactivation.

In the presence of Av1 as an inhibitor, *C. pasteurianum* nitrogenase produces linear rates of acetylene reduction after a short lag phase, the duration of which increases with the amount of Av1 present in the assay mixture (Table 1). Data from experiments in which nitrogenase components were titrated against each other (Tables 1 and 2), when plotted either as total activity (after 5, 10, or 15 min) versus added Av1 or as reaction rates (after lag phase) versus added Av1, all indicate the same amount of Av1 required to eliminate nitrogenase activity. In one experiment, the minimum amount of Av1 required to abolish measurable activity at 15 min had permitted detectable ethylene production by 30, 45, and 60 min. The late activity was linear (about 1 nmol/min) and extrapolated back to 20 min as the time of initiation of ethylene formation. When twice this minimum amount of Av1 was added to Cp nitrogenase, ethylene was first detected at 60 min.

Table 2. Inhibition of *Azotobacter vinelandii* nitrogenase by *Clostridium pasteurianum* Fe protein

Micromolar equivalents				Total nmol of NH_4^+ formed in 10 min
Av1*	Av2*	Cp1*	Cp2*	
0.48	1.25			720
0.48	1.25		0.56	609
0.48	1.25		1.12	300
0.48	1.25		2.20	13
0.48	1.25		4.40	5
0.48	1.25		2.20†	645
0.48	1.25		4.40†	666
0.48	1.25	0.20		579
0.48	1.25	0.40		651
0.48	1.25	0.80		678
0.48	1.25	1.60		684
0.48	1.25	0.40†		699
0.48	1.25	0.80†		732
0.48			2.20	0
	1.25	0.80		0

* See footnote on nomenclature.
† Proteins were O_2-inactivated by exposure to air at 0°.

The inhibition produced by Cp2 contrasts markedly with the inhibition produced by Av1. The rates of ethylene formation are linear through the origin for all levels of Cp2 added (data not shown). When the rates of ethylene formation or the total ethylene formed are plotted versus time, the curve indicates no finite quantity of Cp2 that gives 100% inhibition (Fig. 1A). The first increment of Cp2 has relatively little effect, but then

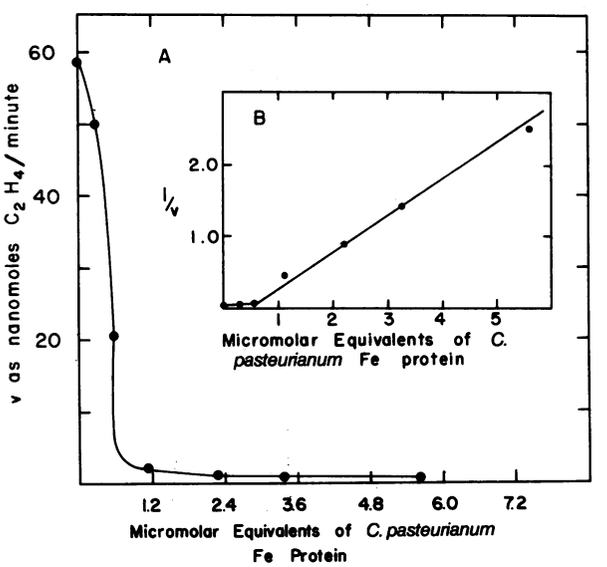

FIG. 1. (A) Activity of *A. vinelandii* nitrogenase as inhibited by *C. pasteurianum* Fe protein. At each inhibitor concentration, the amount of ethylene produced was measured at 5, 10, and 15 min as described in *Materials and Methods* and the rate of ethylene production was determined. The reaction mixtures contained *A. vinelandii* Mo–Fe protein and Fe protein equivalent to 0.2 and 0.9 μM concentrations, respectively. (B) Reciprocal of velocity of *A. vinelandii* enzymatic activity versus concentration of *C. pasteurianum* Fe protein (as inhibitor). Data are replotted from (A). Velocity, v, represents nmol C_2H_4 formed/min. The molar binding ratio of *C. pasteurianum* Fe protein to *A. vinelandii* Mo–Fe protein from this determination is 2.6:1.

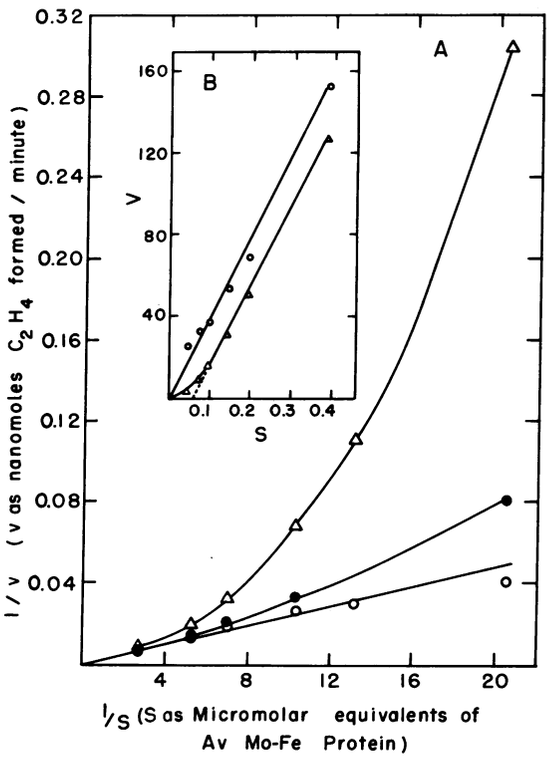

FIG. 2. (A) Double reciprocal plot showing the inhibition of *A. vinelandii* nitrogenase (C_2H_2 reduction) by *C. pasteurianum* Fe protein. The *A. vinelandii* Mo–Fe protein is considered as the variable substrate. The *A. vinelandii* Fe protein equivalent to a 0.89 micromolar concentration was present in all assays. Each point represents the rate from one assay bottle sampled at 5, 10, and 15 min as described in *Materials and Methods*. ○, No *C. pasteurianum* Fe protein; ●, 0.056 micromolar equivalents of *C. pasteurianum* Fe protein; △, 0.112 micromolar equivalents of *C. pasteurianum* Fe protein. (B) Graph of velocity of ethylene formation by *A. vinelandii* nitrogenase plus *C. pasteurianum* Fe protein as inhibitor. The concentration of *A. vinelandii* Mo–Fe protein is expressed in micromolar equivalents and is considered as the substrate for the experiment. Data are replotted from (A). ○, No *C. pasteurianum* Fe protein; △, 0.112 micromolar equivalents of *C. pasteurianum* Fe protein.

the activity decreases rapidly with added Cp2 to less than 10% of the uninhibited rate, whereupon the curve breaks sharply and asymptotically approaches zero. Levels of Cp2 5-fold greater than the amount required to produce 96% inhibition still permit >1% of the original activity (in Fig. 1A, 5.6 μM Cp2 corresponds to a 5-fold molar excess of Cp2 over Av2).

The ratio of Cp2:Av1 at which Av1 gives 100% inhibition, and the extrapolated value at which Cp2 gives 100% inhibition (Fig. 1A), for all experiments performed, ranges between 2 to 3 mol of Cp2 per mol of Av1.

The nature of the inhibitions was not affected by the method used to start the assay. Preincubating the two interacting proteins (Av1 and Cp2) for 1, 2, or 3 min before the addition of the third protein, simultaneously adding the two interacting proteins to a third protein, or preincubating all three proteins in the absence of MgATP (MgATP added to initiate the reaction), all yielded the same results. These treatments were evaluated with Cp2 (as inhibitor of Av nitrogenase) at levels that produced 10% and 97% inhibition and at a level of Av1 (as inhibitor of Cp nitrogenase) which gave 39% inhibition.

Inhibition by Cp2 of Av nitrogenase with Av1 as the variable substrate produces a complex competitive inhibition pattern (Fig. 2A). The same pattern results from Av1 inhibition of Cp nitrogenase, and the existence of the lag in the inhibition of

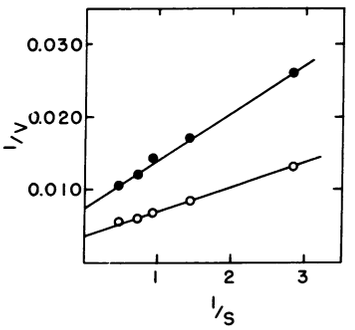

FIG. 3. Double reciprocal plot showing the inhibition of *A. vinelandii* nitrogenase (C_2H_2 reduction) by *C. pasteurianum*. The *A. vinelandii* Fe protein is considered as the variable substrate. The *A. vinelandii* Mo–Fe protein equivalent to a 0.21 micromolar concentration was present in all assays. Values of *v* are expressed as nanomoles C_2H_4 formed per minute and S represents micromolar equivalents of *A. vinelandii* Fe protein. ○, No *C. pasteurianum* Fe protein; ●, 0.46 micromolar equivalents of *C. pasteurianum* Fe protein.

activity does not affect the results (i.e., plotting either total nanomoles C_2H_4 produced or rate after lag). The data for the double reciprocal plots may be interpreted more readily if they are replotted as velocity versus substrate concentration (Av1) (Fig. 2B). Fig. 2B indicates that the inhibition is caused by a tight-binding inhibitor (17). At low concentrations of Av1, Cp2 competes favorably with Av2 to form the inactive complex (2 Cp2:1 Av1) even though Av2 is present in 7-fold molar excess over Cp2. Extrapolation of the linear region of the inhibited curve (Fig. 2B) to the horizontal axis indicates the number of Cp2 proteins bound per Av1 is 1.75. The value obtained for the 0.056 μM level of Cp2 (shown in Fig. 2A, but not in Fig. 2B) is 2.4. A ratio of 2.1 Cp2 per 1 Av1 was derived from the velocity versus substrate plot of Av1 inhibition of Cp nitrogenase. 2 Cp2:1 Av1 inhibits strongly, whereas 1 Cp2:1 Av1 appears much less inhibitory.

Inhibition by Cp2 of Av nitrogenase with Av2 as the variable substrate appears as linear noncompetitive inhibition (Fig. 3). The apparent Michaelis constant for Av2 is unaffected by addition of inhibitory Cp2. The noncompetitive inhibition pattern obtained is expected for a tightly binding competitive inhibitor (17) at the levels of enzyme and inhibitor tested. At very high levels of variable substrate (Av2), the inhibited lines in theory should curve downward and intersect at V_{max} (uninhibited), but experimentally this curvature is difficult to measure.

The Mo–Fe proteins of *Rhodospirillum rubrum, Spirillum lipoferum,* and *Chromatium vinosum* will not crossreact with Cp2 to give an active nitrogenase. However, all three Mo–Fe proteins inhibit acetylene reduction by Cp nitrogenase. Rr2, Sl2, and Cv2 do not crossreact with Cp1 to give an active nitrogenase, nor do they inhibit Cp nitrogenase. Either component of *K. pneumoniae* or *Bacillus polymyxa* yields an active nitrogenase with the complementary component of Cp. Thus, Cp2 interacts with all of the above Mo–Fe proteins, but Cp1 interacts with only Cp2, Kp2, or Bp2.

DISCUSSION

Av1 and Cp2 form a tight, inactive complex even in the presence of an excess of the homologous component, and this complex formation inhibits reduction of N_2 and C_2H_2, oxidation of dithionite, and hydrolysis of ATP—all characteristic reactions of nitrogenase. Whereas Av1 and Cp2 readily form a complex, Cp1 and Av2 do not. Cp2 interacts with the Mo–Fe proteins from several organisms to form inactive complexes. This implies

a greater similarity among the components of the various nitrogenases than was believed previously (18).

Biggins and Kelly (19) suggested that O_2-damaged Fe protein could complex to the homologous Mo–Fe protein and form an inactive complex. The inhibition by a heterologous component requires native protein, as we find that O_2-denatured components do not inhibit. Presumably, O_2 alters the conformation of the binding site and thereby eliminates binding and the resultant inhibition. Eady (2) found O_2-denatured proteins from *K. pneumoniae* did not complex during sedimentation-velocity experiments. Cp2 that was partially inactivated (apparently cold inactivated) had reduced ability to inhibit Av nitrogenase in our experiments.

Av nitrogenase inhibited by Cp2 exhibits linear activity from the initiation of the assay. In contrast, the inhibition of Cp nitrogenase by Av1 is characterized by a lag phase before linear rates of substrate reduction are established. Increasing amounts of Av1 prolong the lag phase and decrease the rate of substrate reduction. Extrapolation of titrations of Cp nitrogenase with Av1 as inhibitor indicates that the same quantity of Av is required to eliminate acetylene reduction activity regardless of the duration of the assay. In contrast, titrations of Cp2 versus Av nitrogenase do not indicate a finite quantity of Cp2 that gives 100% inhibition.

The lack of effect of the various pretreatments on the extent or nature of the inhibition reflects the tight binding between Av1 and Cp2. The inactive complex forms at least as rapidly as the homologous active complex and has a dissociation constant significantly smaller than either homologous complex. The kinetic data indicate that the dissociation constant for Av1–Cp2 is about a tenth that of the dissociation constant for the Av1–Av2 complex and about a hundredth that for the Cp1–Cp2 complex (K_D *C. pasteurianum* nitrogenase $\cong$ 1.0 μM, K_D *A. vinelandii* nitrogenase $\cong$ 0.1 μM, K_D Av1 + Cp2 $\cong$ 0.02 − 0.03 μM). Values of the dissociation constants, as well as the binding ratios, depend on the validity of the assumptions used to calculate molar equivalents. Regardless of the exact values of dissociation constants calculated from these data, the data establish the relative order of the constants.

The $1/v$ versus I plot (cp2 as inhibitor in Fig. 1B) is typical of a tightly binding inhibitor (17). The near zero initial slope at low Cp2 indicates that the first Cp2 is not inhibitory or inhibits very poorly. As the inhibitor increases, only a small increase in the reciprocal velocity occurs until a ratio of 2 to 3 Cp2:1 Av1 is reached, and thereafter $1/v$ increases rapidly and linearly. The extrapolation of this latter part of the curve to the horizontal axis yields the combining ratio of inhibitor molecules to enzyme molecules. However, there may be considerable error in the point of intersection as drawn in Fig. 1B. The initial low slope of the curve indicates that the interaction between Av1 and Cp2 is stronger than the interaction between Av1 and Av2. If the binding of Cp2 to Av1 is infinitely strong, the curve for the $1/v$ versus I plot would have an infinite slope at the combining ratio of Av1 and Cp2. However, the curve has a linear finite slope at inhibitor concentrations greater than the combining ratio, so Av1 and Cp2 must have a finite dissociation constant. The dissociation constant of the Av1:Cp2 complex must be significantly less than the dissociation constant of the Av1:Av2 to exhibit the behavior shown in Fig. 1B. Similar responses are shown in $1/v$ versus I plots when Av1 acts as an inhibitor of *C. pasteurianum* nitrogenase.

The kinetic data of Figs. 1 and 2 indicate two Cp2 proteins bind to one Av1 to form an inactive complex. One mole of Cp2 per mol of Av1 either is unable to inhibit substrate reduction or inhibits very weakly. The addition of the second mol of Cp2 per mol of Av1 markedly inhibits catalysis. The two Cp2 molecules do not interact cooperatively, as this would produce parabolic $1/v$ versus I plots. Neither Fig. 1B nor any other $1/v$ versus I determination produces a parabolic curve.

Cp2 proved to be a noncompetitive inhibitor against Av2 (Fig. 3). A tightly binding inhibitor precludes the direct observation of the competitive interaction expected.

The strong interaction between Av1 and Cp2 relative to homologous components, the kinetic interactions, and the close similarity of all nitrogenases examined suggest that the inhibition by the heterologous component results from binding at the same site or sites as the homologous component. The molar ratio of the homologous components for *C. pasteurianum* (5–7) and soybean nitrogenase (8) has been reported to be 2 Fe proteins: 1 Mo–Fe protein, whereas the molar ratio for *K. pneumoniae* (2) and *A. chroococcum* (3) has been suggested to be 1:1. If Av1 contains two binding sites for Av2, and Cp2 can occupy those same sites, then inhibition is apparent only when both sites on Av1 are occupied by Cp2. This interpretation allows the existence of an active hybrid of Cp2–Av1–Av2. If Av1 contains only one binding site for Av2, which Cp2 also may occupy, then inhibition that requires 2 Cp2:1 Av1 can be explained with a model proposed by Ljones (20). Assuming that Fe protein molecules bound to Mo–Fe protein can accept electrons from other free Fe proteins, then the second Cp2, which produces the inhibition, may bind to the complex (1 Cp1:1 Cp2) while donating electrons to it, and in the process it may trap the first Cp2 to the Av1 in a tight complex.

The data from the several different types of experiments presented yield combining ratios of 2–3 Cp2:1 Av1. Because Cp2 is more labile than Av1 and a slight excess of the inhibitor molecule is required to overcome competition by the homologous component (particularly when Cp2 is the inhibitor), the experimentally determined combining ratios are maximum values. This implies the minimum or real combining ratio is 2 Cp2:1 Av1. This value of 2:1 determined by inhibition with a heterologous component agrees with ratios obtained from homologous components (5–8) and implies that the heterologous component inhibits by binding to the same site as the homologous component.

The two Cp2 molecules do not interact cooperatively. However, the data are consistent with the possibility that the first Cp2 molecule combines tightly to Av1 and does not inhibit or inhibits weakly, whereas the second Cp2 molecule, which also binds tightly, inhibits very effectively.

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