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Carboxyl-Terminal Peptides as Probes for *Escherichia coli* Ribonucleotide Reductase Subunit Interaction: Kinetic Analysis of Inhibition Studies†

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ABSTRACT: The active complex of *Escherichia coli* ribonucleotide reductase comprises two dissociable, nonidentical homodimeric proteins, B1 and B2. When B2 is the varied component, the reductase activity is competitively inhibited by synthetic peptides of varying lengths corresponding to the C-terminus of protein B2. This finding provides the first evidence that the C-terminal peptides and protein B2 share the same binding domain on protein B1. Our data also show that two molecules of peptide can bind to protein B1 with equal affinity. Similar inhibition constants (18 μM) were obtained for peptides containing the C-terminal 20, 30, and 37 residues. When the invariant residue Tyr 356 was omitted, a 2-fold decrease in peptide inhibitory ability was observed. A small peptide, lacking the last 11 residues, had virtually no inhibitory potency. These results, coupled with our previous observations that truncated protein B2, in which one or both polypeptide chains are missing approximately 24 C-terminal residues, had considerably lower or no affinity for B1, suggest that the C-terminal regions are the major determinants in the B1-B2 interaction. In the Appendix, two methods for treatment of kinetic situations pertinent to the ribonucleotide reductase system are presented. One method deals with the determination of kinetic parameters for two components present at comparable levels; the other is concerned with the differentiation of linear and nonlinear competitive inhibition involving the binding of two inhibitor molecules. Both methods should find application to other similar cases.

Ribonucleotide reductase is an essential enzyme for DNA synthesis in all living cells. By reducing ribonucleotides to the corresponding deoxyribonucleotides, the enzyme provides the cells with the precursors needed for DNA synthesis. The *Escherichia coli* enzyme has been extensively studied and has been shown to serve as a prototype for the eukaryotic ribonucleotide reductases. It consists of two nonidentical homodimeric proteins (α_2 and β_2), denoted B1 and B2, respectively [for review, cf. Erikson and Sjöberg (1989)]. Protein B1 contains binding sites for substrates and allosteric regulators and the redox-active cysteines (Reichard, 1988; Åberg et al., 1989; Stubbe, 1989). Protein B2 contributes the tyrosyl radicals and the iron centers (Larsson et al., 1986; Sahlin et al., 1989; Lynch et al., 1989; Nordlund et al., 1990). Proteins B1 and B2 are purified separately, and each is inactive by itself. The active form of the enzyme is the $\alpha_2\beta_2$ complex which can form by a Mg^{2+}-dependent association of B1 and B2.

Little is known about the interaction between subunits in the active complex of ribonucleotide reductase. Evidence from the *E. coli* enzyme suggests that the C-terminal ends of the B2 protein are involved in the interaction with the B1 protein. A heterodimeric form of protein B2, which contains one full-length and one truncated polypeptide chain lacking approximately 24 C-terminal residues,[1] binds poorly to protein B1; the truncated homodimeric form does not bind at all (Sjöberg et al., 1987). Experiments with the enzyme from herpes simplex virus have shown that a nonapeptide representing the C-terminal region of the H2 protein (the viral B2 counterpart) inhibited the viral enzyme activity, but had no effect on the cellular enzyme (Cohen et al., 1986; Gaudreau et al., 1987). This finding indicates that the nonapeptide interferes with the association between H1 and H2. In two recent studies Yang et al. (1990) and Consentino et al. (1990) reported efficient crossinhibition of calf thymus enzyme by

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[1] The number of missing residues in the truncated protein B2 was thought to be 30; it has been redetermined to be 24 (or 22) (M. Karlsson, unpublished results).

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mouse C-terminal peptide and of hamster enzyme by human C-terminal peptide, whereas corresponding *E. coli* and herpes simplex peptides were species-specific.

In the present study, we further strengthen the concept of direct participation of the carboxyl-terminal region in the B1–B2 interaction by demonstrating that the synthetic peptides corresponding to this region serve as competitive inhibitors of protein B2. The potency of peptides of different lengths is also investigated. In addition, we have developed methods for the treatment of kinetic situations that are particular to this enzyme system. The fact that large amounts of B1 and B2 proteins of the bacterial enzyme can be easily and separately obtained from overproducing strains makes this system ideal for detailed kinetic analysis of the mechanism of inhibition by such synthetic peptides.

Experimental Procedures

Materials. [^{3}H]CDP was purchased from Amersham. DTT[2] and bovine serum albumin were obtained from Sigma. *E. coli* thioredoxin and thioredoxin reductase (NADPH) (EC 1.6.4.5) were kindly supplied by Dr. A. Holmgren, Department of Physiological Chemistry, Karolinska Institute, Stockholm, Sweden.

Peptide Synthesis. The different peptides were synthesized by the solid-phase method (Merrifield, 1963), using an Applied Biosystems 430 A synthesizer and the Boc/Bzl protecting group combination (Lindeberg et al., manuscript in preparation). The peptides were purified by HPLC on a polymer-based reversed-phase column kindly provided by Pharmacia (Sweden), using appropriate linear gradients of methanol or acetonitrile in 0.1 M ammonium bicarbonate. Product purity was confirmed by analytical reinjection on the same column and by plasma desorption mass spectrometry (Sundqvist & Macfarlane, 1985). Before use, the peptides were dissolved in 50 mM Hepes buffer, pH 7.6, and quantified by amino acid analysis.

The acetylated peptides BE[12–20] and BE[1–8] were kindly provided by Drs. P. Bartlett and J. Bushweller, Department of Chemistry, University of California, Berkeley.

Purification of Proteins B1 and B2. Proteins B1 and B2 of *E. coli* ribonucleotide reductase were purified from the overproducing strains C600/pLSH1 and C600/pBS1, respectively, as previously described (Sjöberg et al., 1986; Larsson et al., 1988).

Assay of Enzyme Activity. Assays were performed and analyzed as described for the [^{3}H]CDP assay (Thelander et al., 1978) in a final volume of 50 μL with ATP as effector (1.5 mM) and [^{3}H]CDP as substrate (0.5 mM, 18 000–66 000 cpm/nmol) and in the presence of the thioredoxin system (13 μM thioredoxin, 0.5 μM thioredoxin reductase, and 0.4 mM NADPH). In some experiments, the thioredoxin system was replaced by 70 mM DTT and 0.6 mg/mL of bovine serum albumin. Unless otherwise indicated, the concentration of B1 was 0.03 μM, and protein B2 concentrations were varied from 0.03 to 0.7 μM. Enzyme activity is expressed in units (U); one unit represents the production of 1 nmol of dCDP product/min at 25 °C under assay conditions. For the inhibition experiments, the different synthetic peptides were dissolved in 50 mM Hepes buffer, pH 7.6, at about 1 mg/mL, and appropriate amounts added to the assay mixture. Assay of mammalian enzyme (calf thymus M1 and recombinant mouse M2; Mann et al., 1991) was kindly performed by Dr. L. Thelander, Department of Medical Biochemistry and Biophysics, University of Umeå, Sweden.

[2] Abbreviations: DTT, dithiothreitol; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

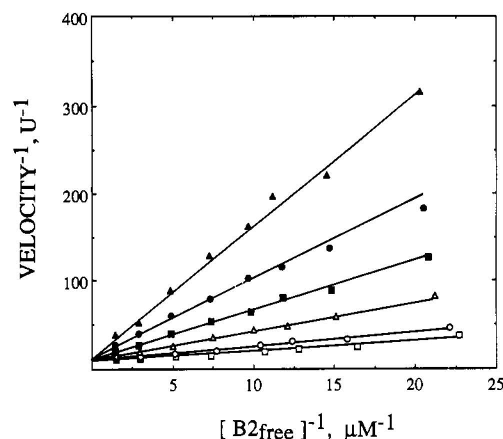

FIGURE 1: Double-reciprocal plot of velocity versus free protein B2 concentrations at different fixed levels of BE[1–20] peptide. Activity was measured as described under Experimental Procedures using 0.0226 μM protein B1. Peptide concentrations: 0 μM (□), 5 μM (○), 15 μM (△), 25 μM (■), 35 μM (●), and 50 μM (▲). Free concentration of B2 was computed according to eq 4. All lines are drawn by using V_m, K_1, and K_{app} values calculated by eqs 5 and 6 (cf. Appendix).

Protein Determination. The concentrations of proteins B1 and B2 were determined from their absorbance at 280 nm minus that at 310 nm. Molar extinction coefficients of 180 000 and 120 000 M^{-1} cm^{-1}, respectively, were used.

Calculation of Kinetic Constants. The inhibition constants (K_i) for the synthetic peptides were determined graphically by plotting $(K_{app}/K_1)^{1/2}$ vs peptide concentration (I) according to eq 15 presented in the Appendix. K_{app} and K_1, Michaelis constants for B2 in the presence and absence of inhibitor, were routinely determined from double-reciprocal plots according to eq 2b, using the total concentration of B2; the required condition of $K_1 \gg \alpha$ was examined by calculating the free concentration of B1 according to eq 3. Whenever necessary, the kinetic parameters (V_m, K_1, K_{app}) were computed either by the more elaborate method described in the Appendix or by computer fitting using the National Institutes of Health MLAB program.

Results

Inhibition of E. coli Ribonucleotide Reductase Activity by Synthetic Peptide BE[1–20]. For a detailed investigation of the mode of inhibition of ribonucleotide reductase with respect to B2, we chose a synthetic peptide corresponding to the last 20 residues in the C-terminal region of B2 protein, BE[1–20] (cf. Table II). Protein B1 concentration was held constant while protein B2 concentrations were varied. Double-reciprocal plots of velocity vs total B2 concentration at five levels of BE[1–20] yielded a family of lines intersecting on the ordinate. In view of the fact that the range of total B2 concentration, 0.05–0.7 μM, was not always much greater than B1 concentration, 0.0226 μM (i.e., total B2 concentration might not approximate free concentration), the data were treated according to the method described in the Appendix to obtain V_m, K_1 (the apparent B1–B2 dissociation constant), and the free B2 concentration. As shown in Figure 1, the resultant double-reciprocal plot again displays a family of lines converging on the y-axis. This pattern is consistent with competitive inhibition, implicating the direct involvement of the C-terminal region of B2 in the B1–B2 subunit binding domain.

Table I presents the kinetic constants obtained by the method given in the Appendix, by curve fitting, or by reciprocal

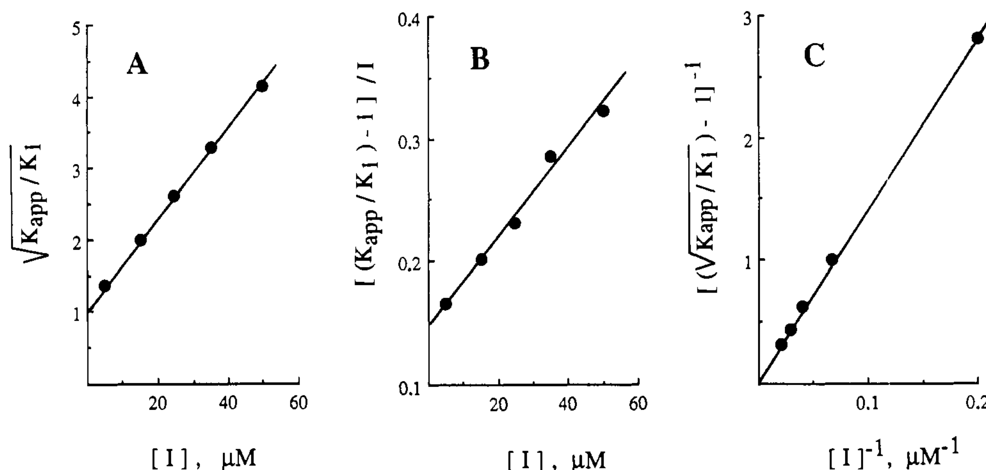

FIGURE 2: Determination of inhibition constants and differentiation between competitive and noncompetitive inhibition by BE[1–20] peptide K_{app} and K_1 were calculated according to eqs 5 and 6 as shown in Table I. Panel A: Competitive inhibition for the case $K_i = K_i'$. Panel B: Competitive inhibition for the case $K_i \neq K_i'$. Panel C: Differentiation between nonlinear competitive and true competitive inhibition. All lines were drawn by the method of linear regression. See Appendix for basis for differentiation.

Table I: Comparison of Kinetic Parameters for the Inhibition of *E. coli* Ribonucleotide Reductase by the BE[1–20] Synthetic Peptide (I)

parameter	method of calculation: equations[a]	MLAB[b]	B2 total[c]
V_m (U)	0.103 ± 0.014	0.101 ± 0.009	0.105
K_1[d] (µM)	0.090 ± 0.020	0.089 ± 0.007	0.122
K_{app} (µM) at			
I = 5 µM	0.165 ± 0.020	0.157 ± 0.006	0.182
I = 15 µM	0.360 ± 0.027	0.351 ± 0.010	0.357
I = 25 µM	0.610 ± 0.069	0.655 ± 0.030	0.625
I = 35 µM	0.974 ± 0.084	1.115 ± 0.046	0.901
I = 50 µM	1.540 ± 0.121	1.803 ± 0.090	1.430

[a] Calculated by using eqs 5 and 6 described in the Appendix. [b] National Institutes of Health curve-fitting program. [c] Calculated graphically by using $1/v$ vs $1/B2_t$ plots. [d] A similar value for the apparent affinity constant of the B1–B2 complex was obtained by using the same kinetic formalism by Binghua and Ehrenberg (personal communication).

plots using total B2 concentrations. The three sets of values are all in good agreement with one another. However, the values of K_1 and K_{app} at low levels of the peptide inhibitor, when determined by using total B2 concentration in reciprocal plots, deviate noticeably from those obtained by the more stringent methods. Nevertheless, the errors so introduced are generally small, especially in the presence of inhibitor. This can be explained by the fulfillment of the condition $K_1 \gg$ free B1 concentration (cf. discussion of eq 2b in Appendix) in most instances. For example, calculation of free B1 concentration for the data reported in Figure 1 using eqs 3 and 9 reveals that, in the absence of inhibitor, the ratio of K_1-to-free B1 concentration increases from a marginal 6.5 to 36 as total B2 concentration increases from 0.05 to 0.7 µM; whereas in the presence of 50 µM BE[1–20], the ratio increases from 77 to 101 in the same total B2 concentration range. In view of the high K_1-to-free B1 ratios in the presence of inhibitor, inhibition constants for other synthetic peptides were routinely determined graphically by using total B2 concentrations.

A Dixon plot of the data shown in Figure 1 yields a series of lines that are concave upward, the concavity becoming more prominent at low B2 concentrations. The results are indicative of the binding of more than one inhibitor molecule to ribonucleotide reductase. This is not unexpected since protein B1 is a homodimer which presumably contains two identical binding sites for the peptide.

Differentiation of Mode of Inhibition and Determination of Inhibition Constants. While the observation of lines intersecting on the ordinate, as shown in Figure 1, is consistent with competition between the BE[1–20] peptide and protein B2 for binding to protein B1, one may not rule out the possibility that the peptide binds to a different site on B1 such that its binding affects only the affinity of B1 for B2 (K_1) but not the catalytic constant (e.g., V_m). The usual intercept and slope replots for testing linear competitive inhibition are valid only for the binding of a single inhibitor molecule. To differentiate between true (linear) competitive inhibition and noncompetitive inhibition without V_m change (nonlinear competitive) where the binding of two interacting or noninteracting inhibitor molecules are involved, we have developed several replots which are described in the Appendix. The kinetic constants given in Table I, column 2, were used to make various diagnostic replots according to eqs 15–17. As shown in Figure 2, panel A, the plot is linear over a 10-fold variation of inhibitor (I) concentration and intersects the ordinate at 1, indicating that BE[1–20] is linearly competitive with respect to protein B2 and the two I molecules have the same inhibition constants, $K_i = K_i'$ = 15.4 µM. In panel B, again, a linear plot over the same I concentration range is obtained, confirming the linear competitive inhibition. The inhibition constants, K_i and K_i' for the two I molecules, are 13.8 and 19.1 µM, which are comparable and suggest little or no interaction (i.e., positive or negative cooperativity) between them on binding to B1. In panel C, the linear plot passing through the origin strongly supports the competitive nature of the inhibition because any appreciable binding of inhibitor to the B1–B2 complex would have resulted in conspicuous x- and y-intercepts. The lack of interaction between the two bound I molecules is also reaffirmed by the linearity of the plot, and the K_i so obtained, 15.8 µM, is in excellent agreement with that determined from panel A, 15.4 µM.

Potency of Varying Lengths of Carboxyl-Terminal Peptides as Inhibitors. Six synthetic peptides corresponding to varying lengths of protein B2 C-terminal region, including the BE-[1–20], were studied under identical experimental conditions. These experiments were intended (1) to see if all peptides have the same inhibition pattern and, if so, (2) to search for any key residue or region responsible for binding to protein B1. All the peptides behaved as competitive inhibitors with respect to protein B2 (except for BE[12–20], see below), as judged by double-reciprocal plots and replots similar to those presented in Figures 1 and 2. The Dixon plots, likewise, were all concave upward. Table II summarizes the inhibition constant for each

Table II: Primary Structure and Inhibition Constants for Protein B2 Carboxyl-Terminal Peptides

name[a]	peptide sequence	K_i[b] (μM)
BE[1-37]	LVSDNVQVAPQEVEVSSYLVGQIDSEVDTDDLSNFQL	18.3
BE[1-30]	VAPQEVEVSSYLVGQIDSEVDTDDLSNFQL	21.5
BE[1-20]	YLVGQIDSEVDTDDLSNFQL	20.0
BE[1-19]	LVGQIDSEVDTDDLSNFQL	40.0
BE[1-8]	Ac-DDLSNFQL	370.0
BE[12-20]	Ac-YLVGQIDSE	4000.0[c]

[a] The numbers in the brackets indicate residues counting from the carboxyl terminus. [b] For the inhibition of ribonucleotide reductase, determined graphically according to eq 15. [c] IC_{50}, concentration of peptide producing 50% of the maximal inhibition. Activity measured with the standard assay containing an equimolar concentration of protein B1 and B2 at 0.032 μM.

peptide. No differences are discernible in their affinity for the B1 protein for BE[1-37], BE[1-30], and BE[1-20] peptides, suggesting that residues from 339 to 355 in the C-terminal end of protein B2 probably are not actively involved in the binding to B1. When Tyr 356 (numbered 20 from the carboxyl end) was omitted in the BE[1-19] peptide, a 2-fold increase in K_i was observed, suggesting that this residue contributes significantly to the B1-B2 interaction or somehow plays a structural role. However, with the BE[12-20] peptide that contains an internal region of the C-terminal end including Tyr 356, the inhibition was too low to permit a rigorous kinetic analysis. The concentration of BE[12-20] needed for 50% inhibition, IC_{50}, determined at a low level of B2, was 4 mM. This value signifies an impaired affinity for this peptide that is at least lowered by 200-fold compared with the other Tyr 356 containing peptides. A 20-fold decrease in affinity was observed for peptide BE[1-8] composed of the last 8 residues of the C-terminal end of B2, suggesting that the very carboxyl-terminal end contains some, though rather weak, determinants responsible for binding to B1.

Effect of the Redox System on E. coli Ribonucleotide Reductase and Its Inhibition by the Synthetic Peptides. In each catalytical turnover, protein B1 is oxidized by substrate and then reduced by thioredoxin present in the assay mixture. To assess the influence of the concentration of the redox system on the enzyme activity measured at variable concentrations of protein B2, we increased the concentration of thioredoxin and thioredoxin reductase. Double-reciprocal plots of velocity vs free B2 concentration at 1- and 7-fold concentrations of the thioredoxin system gave two parallel lines typical of the ping-pong reaction mechanism for B2 and thioredoxin (data not shown). A ping-pong mechanism has also been previously observed with the substrate CDP and thioredoxin (Thelander, 1974). Since either CDP or B2 can combine with B1 in the presence or absence of the other, our finding suggests that the ribonucleotide reductase catalyzed reaction proceeds via a hybrid ping-pong mechanism in which the nucleotide substrate and B2 add randomly to B1. The ping-pong mechanism also means that the concentration of the thioredoxin system employed does not give rise to anomalous effects and hence does not alter the inhibition constants observed with the synthetic peptides even though the K_1 values do vary.

To examine whether the inhibition by the synthetic peptides was due to its interaction with thioredoxin, we used DTT as an alternative to the thioredoxin system. As shown in Table III, inhibition of the enzyme by peptide BE[1-20] was also observed with the DTT redox system, demonstrating that interaction between the peptide and thioredoxin was not the cause of the observed inhibition. However, there was markedly less inhibition in the presence of DTT compared with the thioredoxin system. This phenomenon may arise from the fact that the efficiency of these two redox systems differs, leading to different observed enzyme activity (affecting both V_m and K_1 for B2) in the presence or absence of inhibitor.

Table III: Effect of the Redox System on the Inhibition of *E. coli* Ribonucleotide Reductase by the BE[1-20] Synthetic Peptide

BE[1-20] peptide (μM)	% inhibition[a]	
	thioredoxin system	dithiothreitol[b]
10	24	15
20	45	25
40	65	44

[a] Activity was measured as described under Experimental Procedures, except that 0.074 μM protein B1 and a 4-fold excess of B2 were used. In the absence of peptide, the activities were 0.46 and 0.19 U for the thioredoxin system and DTT, respectively. [b] The standard assay was used except for the substitution of the thioredoxin system by 70 mM DTT and the addition of 0.6 mg/mL bovine serum albumin.

Species Specificity of Peptide BE[1-20] Inhibition. When the BE[1-20] peptide was assayed as inhibitor for the mammalian ribonucleotide reductase, no inhibition was observed at 50 μM peptide, suggesting that the bacterial enzyme inhibition involves a sequence-specific interaction between both the B1 and B2 proteins.

Inhibition of BE[1-20] Peptide versus the Substrate CDP. The mode of inhibition of the peptide BE[1-20] vs CDP was also studied. The patterns obtained by varying the concentration of CDP at several fixed concentrations of peptide are consistent with a noncompetitive type of inhibition. The results agree with the reported noncompetitive inhibition of the herpes simplex virus enzyme by the nonapeptide vs CDP (Cohen et al., 1986) and indicate that the substrate binding site does not overlap with the carboxyl-terminal binding domain.

Discussion

The mechanism of inhibition of ribonucleotide reductase by carboxyl-terminal synthetic peptides has not been fully understood. It is known from the herpes simplex virus enzyme that a nonapeptide corresponding to the carboxyl terminus of viral protein H2 inhibits the enzyme activity and binds to protein H1 (Paradis et al., 1988). It has also been proposed that, since this enzyme, like the *E. coli* enzyme, undergoes subunit dissociation, the nonapeptide can gain access to the H2 binding site on H1 (Darling et al., 1990). However, no conclusive data were available. The results presented here represent the first kinetic evidence that the carboxyl-terminal peptides and protein B2 share a common binding region on *E. coli* protein B1.

To reach the above conclusion, we have used peptides corresponding to the carboxyl-terminal end of the bacterial ribonucleotide reductase protein B2 as probes for studying the subunit interaction. The peptides were synthesized to contain different parts of the regions of B2 that supposedly constitute the interface between B1 and B2. We found that the peptides specifically inhibit the enzyme activity with different potencies (Table II), depending upon the peptide sequence. Our results are in agreement with the inhibition by H2 carboxyl-terminal peptides first reported for the herpes simplex virus enzyme (Dutia et al., 1986) and suggest a general mechanism for the inhibition by carboxyl-terminal peptides of class I ribonucleotide reductase which is present in almost all eukaryotes including mammals.

The most important observation in the present work is the finding that all of these peptides behave as competitive in-

Table IV: Amino Acid Sequence Alignment for the Carboxyl-Terminal Region of Protein B2 from Several Species[l]

	343	353	363	373	ref
E.coli	WINTWLVSDN VQVAPQ E	VEV SS Y	LVGQIDS	EVDTDDLSNF QL....	a
Phage T4	WIREYLNSDN VQSAPQ E	VEL SS Y	LVAQIDN	DVDDKVMMSF KKYF...	b
Clam	......ISLE GKTNFF E	KRV GE Y	QKMGVMS	GGNTGDSHA. FTLDADF	c
Urchin	.....TISLE GKTNFF E	KRV GE Y	QLMGVMS	STNSSKKQHT FSLDEDF	c
Mouse	.....NISLE GKTNFF E	KRV GE Y	QRMGVMS	NSTENS.... FTLDADF	d
Yeast	.....NISLA GKTNFF E	KRV SD Y	QKAGVMS	KSTKQEAGA. FTFNEDF	e,f
Vaccinia	.....NISLE GKTNFF E	KRV GE Y	QKMGVMS	QEDNH..... FSLDVDF	g
Herpes	....SLMSTD KHTNFF E	CRS TS Y	AGAVVND	L.........	h,i
Varicella	....AFMIAD KNTNFF E	RHS TS Y	AGTVIND	L.........	j
Epstein-Barr	.....YMTSI KQTNFF E	QES SD Y	TMLVVDD	L.........	k

[a] Carlson et al. (1984). [b] Sjöberg et al. (1986). [c] Standart et al. (1985). [d] Thelander and Berg (1986). [e] Elledge and Davis (1987). [f] Hurd et al. (1987). [g] Slabaugh et al. (1988). [h] McLaughlan and Clements (1983). [i] Swain and Galloway (1986). [j] Gibson et al. (1986). [k] Davidson et al. (1986). [l] Residues that are present in at least five places in addition to the *E. coli* sequence are indicated by a black background, and if present in all species, they are boxed. The numbering refers to the *E. coli* sequence.

hibitors versus protein B2. The most straightforward interpretation of the inhibition is that protein B2 and the peptides bind to the same domain on protein B1. The competitive nature of the inhibition was established after subjecting the data to a rather stringent test using various diagnostic plots. Moreover, a method has also been developed to correct for errors introduced by the marginal B2 to B1 ratio (see Appendix). In fact, in one experiment (data not shown) we purposely increased the total B1 concentration 5-fold to examine the effectiveness of such treatment and obtained a corrected K_1 value which is identical with the K_1 obtained at the usual B1 concentration. The deviation of K_1 determined by using total B2 concentration in a double-reciprocal plot and by other methods which in essence use the free B2 concentration can be explained from another angle. K_1 should equal the *free* concentration of B2 (the varied component) at $V_m/2$ where half of the total B1 is in the B1–B2 complex form. The *total* B2 concentration at half-saturation, then, is equal to K_1 plus half of the total B1 concentration. Therefore, the difference between the two K_1 values so determined, in theory, amounts to half of total B1 concentration.[3]

Our results with five C-terminal peptides also indicate that both binding sites on B1 have the same affinity for a given inhibitor. All the data are amenable to treatment with plots used in Figure 2, panels A and C, in which a common K_i for both sites is assumed. When the plot in Figure 2, panel B, was used, the values of K_i and K_i', inhibitor constants for the first and second binding sites, were generally comparable and showed no trends as to which site was the tighter one. The average Hill coefficient for the five peptides listed in Table II is 1.00 ± 0.15. The observation indicates that the two bound peptides do not interact with each other on protein B1. The possibility that the two C-terminal regions can communicate through a protein B2 or B1–B2 complex, however, cannot be excluded. A similar situation with noncooperative binding sites on protein B1 was reported earlier by von Döblen and Reichard (1976) for the interaction with substrates and effectors.

The K_i values determined by us should reflect the sizes of dissociation constants of the inhibitors. Of the C-terminal peptides tested, the strongest inhibitors are BE[1–37], BE-[1–30], and BE[1–20], with a K_i of about 20 μM. The value of K_1 may not be a true dissociation constant, but it should be a reasonable approximation for the magnitude of the B1–B2 dissociation constant. Although the size of K_1 increases with the concentration of thioredoxin and thioredoxin reductase to a limiting value of $\sim$0.18 μM, this is only about twice the value of $\sim$0.1 μM given in Table I. Thus, the affinity of the C-terminal peptides for protein B1, as far as we have examined, is roughly 100-fold lower than that of protein B2. This comparison seems to suggest that regions of B2 other than the C-terminus play a pivotal role in the subunit interaction. However, it should be pointed out that protein B2 is a dimer containing two C-terminal regions that interact with B1. Conceivably, the increased binding energy due to dual contacts, much like the increased chelating effect of a bidentate, can readily account for the observed difference. Furthermore, previous experiments with truncated protein B2 (Sjöberg et al., 1987) showed that the heterodimer, with one polypeptide chain missing approximately 24 C-terminal residues, binds poorly to B1, whereas the doubly truncated homodimer does not bind at all. This observation shows that, in the absence of the C-terminal residues, other regions of B2 do not contribute to B1–B2 subunit interaction. It also shows that a single native β polypeptide chain contributes little to the interaction. In the recently resolved X-ray crystal structure of *E. coli* protein B2 (Nordlund et al., 1990) the last 32 residues of the carboxyl end could not be defined. This finding suggests that the C-terminal is highly flexible and that the truncated B2 polypeptide chain probably retains the same structure as

[3] As an example, consider the K_1 and K_{app} values of 0.122 and 0.182 μM (at $I = 5$ μM) given in Table I, column 4. After subtracting half of the total B1 concentration (0.015 μM) from them, we obtain 0.107 and 0.167 μM, respectively, which approach the values of 0.09 and 0.165 μM given in Table I, column 2. The differences in K_i values for peptide BE[1–20] obtained in separate experiments (Figure 2 and Table II), 15 and 20 μM, can be explained in a similar manner. When the corrected K_1 is used for computing the K_i for Table II, the K_i value of 20 μM originally obtained by using total B2 concentration reduces to 18 μM. Consequently, caution should be exercised when an interaction constant is estimated from the total concentration of the varied component at half-saturation. It is not uncommon to find in the literature that a "constant" so determined often merely reflects half the total enzyme (or the fixed component) concentration employed.

the bulk of the protein. In this regard, our present data clearly show that the C-terminal region of B2 is the major determinant in its interaction with B1. It is tempting to speculate that this C-terminal end forms a separate domain which via a hinge region connects to the rest of B2.

On the basis of the crystal structure of *E. coli* protein B2, Nordlund et al. (1990) have proposed that the upper part of the heart structure of the folded dimer is the interaction area with B1. Partially or highly conserved residues among protein B2 from different species, namely, Trp 48, Glu 52, Asp, 58, Tyr 307, Arg 236, and Ser 341, are located in this area. The C-terminal region of protein B2 has also been proposed to be located at this side of the molecule. Table IV shows the alignment of the C-terminal part of protein B2 and nine other equivalent sequences, where only two invariant residues, Tyr 356 and Glu 350, are found. We did not find any differences in peptide binding relating specifically to the presence or absence of the invariant Glu 350. Therefore, it is plausible that the segment around this residue is not directly involved in the binding to protein B1. Note that the longer peptide (BE[1-37]) covers a large area upstream of the invariant Tyr 356, which also includes the two partially conserved Ser 341 and Val 353 residues (Table IV).

On the other hand, an effect on the affinity for B1 was discernible when Tyr 356 was excluded as in peptide BE[1-19]. The 2-fold increase in the K_i for the bacterial enzyme upon elimination of Tyr 356 is less than the 5- to 8-fold increase of the IC_{50} reported by Gaudreau et al. (1987) for the herpes simplex virus enzyme when the conserved Tyr was eliminated from the nonapeptide. However, the compositions of the two peptides are different; the carboxyl-terminal end in *E. coli* has an extension of 20 residues compared with the 9 residues in the viral enzyme. The binding domains on proteins B1 or H1 undoubtedly are also different, as evidenced by the species-specific inhibition shown by the peptides. Although a systematic study of the more distal parts of the carboxyl-terminal end of the bacterial B2 protein has not been performed [see also Bushweller and Barlett (1991)], a K_i of 370 μM for the peptide corresponding to the last eight residues indicates that this region is involved. This is further supported by the almost complete absence of inhibitor effect in peptide BE[12-20] that still contains the conserved Tyr but lacks this particular C-terminal part. It should be noted that both BE[1-8] and BE[12-20] synthetic peptides are acetylated, and one may wonder whether the acetylation results in reduced affinity. In view of the studies of Gaudreau et al. (1990) on the inhibition by C-terminal peptides of the viral enzyme, however, this is unlikely since the acetylated peptides are generally equal or better inhibitors relative to their unacetylated analogues. Studies by means of site-directed mutagenesis of the conserved residues in the carboxyl-terminal end of protein B2, currently in progress in our laboratory, should allow further insight into their roles in the subunit interaction in the active complex.

In conclusion, the present kinetic study on the inhibition of ribonucleotide reductase by carboxyl-terminal peptides has deepened our understanding of the mechanism of inhibition by these peptides. It provides the strongest evidence for the participation of the C-terminal end of B2 in the ribonucleotide reductase subunit interaction.

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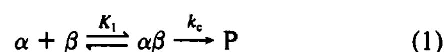
APPENDIX

In this section, two problems related to the treatment of kinetic data for the ribonucleotide reductase system are addressed. The methods described here, however, should find application to other similar situations.

Determination of Kinetic Parameters for Tight-Binding Two-Component Systems. Kinetic analysis of enzyme activation by a tightly bound component (or a component present at comparable levels), e.g., the B1-B2 protein interaction of ribonucleotide reductase or the calmodulin-stimulated enzymes, can be rather complicated. The total concentration of the varied component usually no longer approximates the free concentration, thereby rendering the usual kinetic plots inapplicable. The following is an approach which we employ to treat this type of problem. For the activation of protein B1 (α) upon complexation with protein B2 (β),[4] we can write



$$\alpha + \beta \underset{}{\overset{K_1}{\rightleftharpoons}} \alpha\beta \xrightarrow{k_c} \mathrm{P} \quad (1)$$

where both α and $\alpha\beta$ are saturated with substrate (as is the case with our experimental conditions) and k_c and P denote the catalytic rate constant and the product. To simplify the notations, we use α and β, rather than α_2 and β_2, to indicate the concentrations of B1 and B2. Note that substrate saturation is a condition for the simplification, not validity, of the derivations to follow.

If we keep B1 constant and vary B2, we have, from equilibrium treatment, that

$$v = \frac{V_m \beta}{K_1 + \beta} \quad (2a)$$

$$v = \frac{V_m \beta_t}{(K_1 + \alpha) + \beta_t} \quad (2b)$$

where $V_m = k_c \alpha_t$ and the subscript t indicates total, rather than free, concentration. Equation 2b shows that, when $K_1 \gg \alpha$, the kinetic parameters can be determined from the usual $1/v$ vs $1/\beta_t$ plot (from eq 2b) without suffering much loss of accuracy. α can be estimated from the equation

$$\alpha = \alpha_t(1 - v/V_m) \quad (3)$$

If V_m can be conveniently and precisely measured at a saturation level of B2, then the free concentration of B2 can be calculated from the relationship

$$\beta = \beta_t - \alpha_t(v/V_m) \quad (4)$$

and the kinetic data treated according to eq 2a.

When V_m is not known (or not routinely determined), as in our case, we analyze the data in the following fashion: Substituting eq 3 into eq 2b and rearranging, we have

$$K_1 = \beta_t[(V_m/v) - 1] - \alpha_t[1 - (v/V_m)] \quad (5)$$

Thus, from two velocities v_1 and v_2 measured at two total B2 concentrations, β_{t1} and β_{t2}, we obtain

$$K_1 = \beta_{t1}[(V_m/v_1) - 1] - \alpha_t[1 - (v_1/V_m)]$$
$$= \beta_{t2}[(V_m/v_2) - 1] - \alpha_t[1 - (v_2/V_m)]$$

[4] Note that the α and β pair is analogous to either the enzyme-substrate or enzyme-activator case. Thus, when α represents an enzyme having a basal activity (v_0) in the absence of the activator β, the approach presented here is valid provided Δv (=$v - v_0$ in the presence or absence of inhibitor) is used in the analysis. All the equations (1-17) remain valid on substituting v and V_m with Δv and ΔV_m, respectively.

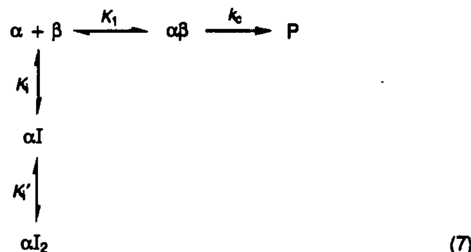
or

$$V_m = \{(\beta_{t1} - \beta_{t2}) + \sqrt{(\beta_{t1} - \beta_{t2})^2 - 4\alpha_t(v_1 - v_2)[(\beta_{t1}/v_1) - (\beta_{t2}/v_2)]}\}/\{2[(\beta_{t1}/v_1) - (\beta_{t2}/v_2)]\} \quad (6)$$

The average value of V_m determined from various β_t and v pairs (after discarding the extreme values) is then used to compute K_1 from eq 5 and β from eq 4.

Alternatively, both V_m and K_1 are fitted according to eq 5 by the National Institutes of Health MLAB computer curve-fitting program. V_m and K_1 for the same set of data are usually calculated by the above methods and their values compared. In many cases, especially when the peptide inhibitors are present (i.e., the free concentration of B1 is reduced), the use of eq 2b to analyze data graphically is quite adequate. It should be noted that the approach described here is not limited to enzyme activity measurement. Any measurable signal specific for the amount of complex formed can be used in its stead.

Determination of Inhibition Constants and Differentiation of True or Apparent Competitive Inhibition for an Inhibitor with Two Binding Sites. Both the B1 and B2 proteins of ribonucleotide reductase are dimeric proteins. Therefore, 2 mol of C-terminal peptides should be able to combine with 1 mol of B1. If the C-terminal region of B2 is indeed involved in the B1–B2 interaction, one would expect the peptide inhibitor (I) to compete with B2. Presumably, the binding of one I molecule to B1 is sufficient to block the binding of the dimeric B2. The competitive inhibition model can be written as



(7)

Equilibrium treatment yields equations similar to eqs 2a, 2b and 3–5:

$$v = \frac{V_m\beta}{K_{app} + \beta} = \frac{V_m\beta_t}{K_{app} + \alpha\left(1 + \frac{2I}{K_i} + \frac{I^2}{K_iK_i'}\right) + \beta_t} \quad (8)$$

$$\alpha = \alpha_t[1 - (v/V_m)]/[1 + (2I/K_i) + (I^2/K_iK_i')] \quad (9)$$

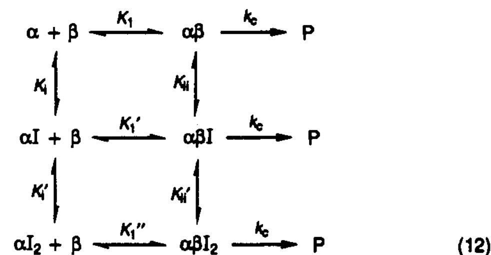
$$\beta = \beta_t - \alpha_t(v/V_m) \quad (4)$$

$$K_{app} = K_1[1 + (2I/K_i) + (I^2/K_iK_i')] \quad (10)$$

$$K_{app} = \beta_t[(V_m/v) - 1] - \alpha_t[1 - (v/V_m)] \quad (5)$$

$$K_{app} = K_1[1 + (I/K_i)]^2 \qquad \text{if } K_i = K_i' \quad (11)$$

Clearly, the methods described in the foregoing section are equally applicable to the competitive inhibition studies. Although the C-terminal peptide inhibition exhibits reciprocal plots with a family of lines intersecting on the ordinate (see Figure 1), one may not conclude that the inhibition is competitive. The possibility exists that the peptide inhibitor binds to B1 at a site different from the B2 binding site, but its presence affects only the affinity of B1 for B2 (K_1), not its V_m. This type of noncompetitive inhibition is sometimes referred to as the nonlinear competitive case:



(12)

Equilibrium treatment leads to equations identical in form with eqs 8–10 and 4 except that

$$K_{app} = \frac{K_1\left(1 + \frac{2I}{K_i} + \frac{I^2}{K_iK_i'}\right)}{\left(1 + \frac{2I}{K_{ii}} + \frac{I^2}{K_{ii}K_{ii}'}\right)} \quad (13)$$

$$K_{app} = \frac{K_1\left(1 + \frac{I}{K_i}\right)^2}{\left(1 + \frac{I}{K_{ii}}\right)^2} \qquad \text{if } K_i = K_i' \text{ and } K_{ii} \text{ and } K_{ii}' \quad (14)$$

To differentiate among the four possible choices represented by eqs 10, 11, 13, and 14, we use the following replots:

Case Ia: Competitive Inhibition, $K_i = K_i'$. From eq 11, we have

$$\sqrt{\frac{K_{app}}{K_1}} = 1 + \frac{I}{K_i} \quad (15)$$

A plot of $(K_{app}/K_1)^{1/2}$ vs I will yield $-K_i$ as the x-intercept, and the line will pass through 1 on the ordinate.

Case Ib: Competitive Inhibition, $K_i \neq K_i'$. By rearrangement of eq 10, we have

$$\left(\frac{K_{app}}{K_1} - 1\right)\frac{1}{I} = \frac{1}{K_i}\left(2 + \frac{I}{K_i'}\right) \quad (16)$$

A plot of $[(K_{app}/K_i) - 1]/I$ vs I will yield $2/K_i$ as the y-intercept and $-2K_i'$ as the x-intercept. For perfect data, the plot used in case Ib should give $K_i = K_i'$ for case Ia.

Case IIa: Noncompetitive, V_m Unaffected, $K_i = K_i'$ and $K_{ii} = K_{ii}'$. Rearrangement of eq 14 yields the expression

$$\frac{1}{\sqrt{K_{app}/K_1} - 1} = \left(\frac{K_i}{K_{ii} - K_i}\right)\left(1 + \frac{K_{ii}}{I}\right) \quad (17)$$

A plot of $1/[(K_{app}/K_1)^{1/2} - 1]$ vs $1/I$ will yield $-1/K_{ii}$ as the x-intercept, and K_i can be calculated from the y-intercept, $K_i/(K_{ii} - K_i)$. Note that with this plot, when applied to cases Ia and Ib, the line may be linear (for case Ia) and both should pass through the origin. Thus, this plot is best suited for distinction between linear and nonlinear competitive inhibition.

Case IIb: Noncompetitive, V_m Unaffected, $K_i \neq K_i'$, and $K_{ii} \neq K_{ii}'$. If K_i, K_i' and K_{ii}, K_{ii}' are sufficiently different, the plots used in cases Ia, Ib, and IIa should all become curvilinear when applied to case IIb.

In all cases, a good linear fit (or deviation from it) over a sufficient range of I concentration for the plots described here can be taken, like replots in the conventional inhibition studies, as support (or rejection) for the respective mechanisms.

Registry No. Be[1–37], 133227-71-1; Be[1–30], 133128-08-2; Be[1–20], 133101-37-8; Be[1–19], 133128-09-3; Be[1–8], 133101-

38-9; Be[12-20], 133101-39-0; ribonucleotide reductase, 9040-57-7.

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