Multiple Binding Sites of Carboxypeptidase B: The Evaluation of Dissociation Constants by Quantitative Affinity Chromatography†

Jean Danner,‡ Joan E. Somerville, Julia Turner, and Ben M. Dunn*

ABSTRACT: Multiple binding sites for carboxypeptidase B (CPB) were studied by quantitative affinity chromatography. In the presence of 25 mM imidazole at pH 6.4, the enzyme was retarded by a gel to which D-Phe had been covalently bound (agarose–PheOH). Neither agarose–D-Phe methyl ester nor an agarose derivative that contained free carboxylate groups (Affi Gel 201) retarded the protein. D-Phe and *N*-acetyl-D-Phe but not Gly competitively eluted CPB. The dissociation constants (Dunn, B. M., & Chaiken, I. M. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2382–2385) for the CPB·D-Phe and CPB·*N*-acetyl-D-Phe complexes were found to be 9.8 mM and 7.8 mM, respectively. The binding of the enzyme to agarose–PheOH was 7.5 times stronger than to the soluble D-Phe, suggesting that the spacer arm and/or agarose matrix was a contributing factor in the binding process. β-Phenylpropionic acid and ε-aminohexanoic acid induced further retardation of CPB on agarose–PheOH. By using a generalized binding equation, dissociation constants for the binary complexes CPB·ε-aminohexanoic acid and CPB·β-phenylpropionic acid in 25 mM phosphate at 6.4 were calculated as 1.8 and 0.65 mM, respectively. The dissociation of the ternary complexes (CPB·modifier·immobilized D-Phe) into the respective binary complexes plus immobilized D-Phe proceeded with K_D's = 0.32 and 0.98 mM, respectively. In the presence of ε-aminohexanoic acid in phosphate buffer, pH 6.4, no induced retardation was observed on a column that contained free carboxylates but no Phe. The retardation induced by ε-aminohexanoic acid increased with decreasing pH. The results suggest that binding is facilitated by the presence of an acidic form of the amino acid side chain on CPB.

Carboxypeptidase B (EC 3.4.12.3) and carboxypeptidase A (EC 3.4.12.1), C-terminal exopeptidases, differ in their specificities, the former hydrolyzing bonds bearing a C-terminal basic residue and the latter acting on bonds bearing aromatic and neutral amino acids (Hartsuck & Lipscomb, 1971; Folk, 1971). Additional studies, however, have demonstrated that CPB[1] shares with CPA nearly homologous sequences in the active site regions (Titani et al., 1975) and a binding site or sites for aromatic amino acid residues (Wintersberger et al., 1962; Moore & Benoiton, 1972; Zisapel & Sokolovsky, 1972). Multiple binding modes have been suggested for both carboxypeptidases (Vallee et al., 1968; Alter & Vallee, 1978; Moore & Benoiton, 1975; Ager & Haas, 1977) and, in the case of CPB, models have accommodated different sites for basic and aromatic residues (Akanuma et al., 1971; Zisapel & Sokolovsky, 1975).

We chose to investigate quantitatively the binding modes of CPB by the technique of quantitative affinity chromatography (Dunn & Chaiken, 1974). The method is based on the equilibria that exist between a soluble enzyme and immobilized ligand and has been successfully used for the direct determination of binding constants for staphylococcal nuclease (Dunn & Chaiken, 1974), ribonuclease (Chaiken & Taylor, 1976), and chymotrypsin (Gilbert, 1978). Using a generalized form of a binding equation we have not only determined the dissociation constants of soluble enzyme modifier complexes, but we have also calculated those of immobilized ternary complexes.

Experimental Section

Materials

Carboxypeptidase B (chromatographically purified), hippuryl-L-Arg, εAHx, Tris, imidazole (low fluorescence blank), D-Arg, *N*-acetyl-L-Arg, *N*-acetyl-D-Phe, Tes, and EDAC were purchased from Sigma Chemical Corp. Two lots of CPB were used in this study. Lot 86C-8005 with specific activity of 90 units/mg was used for preliminary experiments. Lot 114C-8120 with specific activity of 185 units/mg was used for all experiments reported here. In each preparation, there is a small amount of protein that does not bind to the affinity matrix and thus emerges at the breakthrough point. The amount of this is higher in lot 86C-8005. Since this provides a convenient, noninterfering marker that is rapidly separated from active CPB, we have not removed it. D-Phe and D-Phe methyl ester hydrochloride were products of Vega-Fox and Lys·HCl was obtained from Mann Research Lab. βPP was purchased from Eastman Kodak Co. Affi Gel 201 was purchased from Bio-Rad.

Equipment

Ultraviolet spectra were taken on a Cary 15 spectrophotometer. Enzyme assays were carried out on a Gilford 250 spectrophotometer equipped with a Gilford 6050 recorder and Lauda K-2/R circulating water bath. Titrations were performed with a Radiometer autoburette (Model ABU-12) equipped with a Radiometer pH meter (PHM 64), titrator (TTT 60), and servograph (REC-61). When column fractions were monitored by UV absorbance an Isco Absorbance Monitor (Model 226) equipped with a Bio-Rad strip chart recorder (Model 1310) was employed. Amino acid analysis was performed on a Beckman Model 120C amino acid analyzer.

Methods

Synthesis of the Affinity Absorbant. In a typical synthesis, 20 mL of Affi Gel 201 was suspended in dioxane–H_2O (4:1, v/v). D-Phe methyl ester hydrochloride (1.03 mmol) was added and the pH meter reading (standardized with aqueous

† From the Department of Biochemistry and Molecular Biology, JHM Health Center, University of Florida, Gainesville, Florida 32610. *Received November 8, 1978.* Supported by a research grant from the National Institutes of Health, Grant No. GM 22421. A preliminary account of this work was presented to the 69th annual meeting of the American Society of Biological Chemists, Atlanta, GA, 1978.

‡ Present address: National Institute for Occupational Safety and Health-ALOSH, Morgantown, WV 26505.

[1] Abbreviations used: CPB, carboxypeptidase B; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; εAHx, ε-aminohexanoic acid; βPP, β-phenylpropionic acid (hydrocinnamic acid); Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Tes, *N*-tris(hydroxyethyl)methyl-2-aminoethanesulfonic acid.

0006-2960/79/0418-3039$01.00/0

buffers) was adjusted to 6.0 with a few drops of 1 M NaOH. EDAC (5 mmol) in about 2 mL of water was added to the stirred suspension and the contents were kept at room temperature. The pH was maintained at 6.0 by the autotitrator with 0.1 N HCl. After the consumption of protons had ceased (about 40 min), the reaction vessel was disconnected from the autotitrator, placed in a hood, and allowed to remain with stirring overnight. The gel was filtered and washed in order with large amounts of H_2O, 0.1 M NaCl, H_2O, 0.1 M HAc, and H_2O. The washed gel was stored at 4–5 °C in the presence of buffer containing 0.02% NaN_3. The gel thus prepared will be referred to as agarose–PheOMe.

Agarose–PheOMe was saponified as follows. The gel was suspended in 2.7 volumes of absolute CH_3OH. An amount of 0.05 M NaOH equivalent to 2 mequiv per mequiv of carboxyl groups on the original uncoupled gel was added. The pH of the resulting gel suspension was 11.9 when measured with a glass electrode standardized with aqueous standard buffers. After refluxing for 5 h, the contents were filtered and washed as described for agarose–PheOMe. The filtrate had a slightly pink tint. The saponified gel, referred to hereafter as agarose–PheOH, was stored in buffer at 4–6 °C in the presence of 0.02% NaN_3.

Ultraviolet Spectra of the Gels. A small amount of gel was placed in a graduated centrifuge tube and spun briefly in a clinical centrifuge to separate it from the storage buffer. After the buffer solution was removed, water was added to the packed gel, and the contents were mixed and spun again. This process was repeated twice. The volume of packed gel was read directly and suspended in a solvent of glycerol–50 mM Tris-HCl, pH 7.5 (1:1, v/v), such that the final volume represented a 10-fold dilution of the gel. Immediately after mixing, the gel was scanned in the UV against the glycerol–Tris reference. The absorbance at 300 nm was subtracted from that at 258 nm and the concentration of bound Phe calculated on the basis of an E_{258} = 206 for free Phe (Greenstein & Winitz, 1961).

Titrations of the Gels. The gel sample was acidified with 1 N HCl and washed with water until the pH of the wash was no longer acidic. The gel was then washed with 0.1 M NaCl and a measured volume (about 1 mL) suspended in the salt solution (about 13 mL).

Titrations were performed under N_2 with stirring and aliquots of NaOH (15–20 μL) were added by the autotitrator. Subsequent additions were made after the pH reached a constant value which usually took 3–5 min.

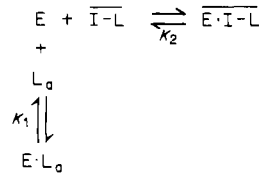
Amino Acid Analysis. Samples were hydrolyzed in vacuo in 6 N HCl at 114 °C, for 24 h.

Carboxypeptidase B was assayed by the procedure outlined by Wolff et al. (1962). One milliliter of 1 mM hippuryl-L-arginine in 25 mM Tris-HCl, pH 8, was equilibrated at 30 °C and the reaction was initiated by addition of the enzyme. Activity was monitored at 254 nm at 30 °C and has been defined as ΔA_{254} min^{-1} mL^{-1}.

Affinity Chromatography. The enzyme (supplied as a solution in 0.1 M NaCl) was dialyzed against the buffer to be used in the chromatographic experiment. Chromatography was carried out at ambient temperature. The fractions were monitored by either activity assay or absorbance at 280 nm with the absorbance monitor chart recorder assembly. Dialysis did not significantly lower the activity of CPB.

Quantification of Dissociation Constants from Chromatographic Data. Elution volumes were measured as the maximum of plots of CPB activity vs. fraction number. Recovery of applied activity was always greater than 70% and was constant throughout each series of experiments. This implies that any influence of eluant on activity is diluted out in the assay procedure. In the case where *competitive elution* was observed, the data were analyzed by the procedure of Dunn & Chaiken (1974). The scheme and equation which they employed are given in eq 1, where E = applied protein;

Competitive Elution



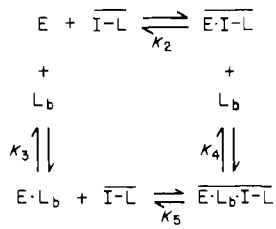
$$\frac{1}{V_e - V_0} = \frac{K_2[L_a]}{K_1(V_0 - V_m)[\overline{I\text{-}L}]} + \frac{K_2}{(V_0 - V_m)[\overline{I\text{-}L}]} \quad (1)$$

L_a = soluble ligand that competitively elutes the protein; $\overline{\text{I-L}}$ = immobilized ligand; $E·L_a$ = mobile phase protein·ligand complex; $\overline{\text{E·I-L}}$ = stationary phase protein·ligand complex; V_e = elution volume of the protein; V_m = mobile phase volume; V_0 = elution volume in the absence of interaction between protein and ligand.

$$K_1 = \frac{[E][L_a]}{[E·L_a]};\ K_2 = \frac{[E][\overline{I\text{-}L}]}{[\overline{E·I\text{-}L}]}$$

The dissociation constants for ternary complexes were obtained from *ligand induced retardation* data. Under these conditions, the presence of the ligand in the equilibrating and eluting buffer results in increased retardation of the protein compared to that observed in its absence. A scheme and equation that describes the effect are given in eq 2, where L_b

Ligand-Induced Retardation



$$V_e = V_0 + \frac{(V_0 - V_m)\left[\frac{[\overline{I\text{-}L}]}{K_2} + \frac{[L_b][\overline{I\text{-}L}]}{K_3K_5}\right]}{1 + \frac{[L_b]}{K_3}} \quad (2)$$

= mobile phase ligand that enhances the binding of E to $\overline{\text{I-L}}$ through the formation of the stationary phase ternary complex, $\overline{\text{E·L}_b\text{·I-L}}$.

$$K_3 = \frac{[E][L_b]}{[E·L_b]};\ K_4 = \frac{[L_b][\overline{E·I\text{-}L}]}{[\overline{E·L_b·I\text{-}L}]};\ K_5 = \frac{[E·L_b][\overline{I\text{-}L}]}{[\overline{E·L_b·I\text{-}L}]}$$

The remaining symbols have been defined as for competitive elution.

In the scheme for ligand induced retardation, the ternary complex, $\overline{\text{E·L}_b\text{·I-L}}$, can be reached by either the K_2–K_4 or K_3–K_5 pathway. Although we are able to calculate the dissociation constants for either wing, we cannot distinguish which path predominates in our experiments. We have, therefore, arbitrarily chosen the K_3–K_5 pathway because data exist for kinetically derived K_3 values which may be compared with our determinations. Hence, using this pathway, we are able to determine the validity of our results. When K_2 is so large that retardation does not occur in the absence of added ligand, the

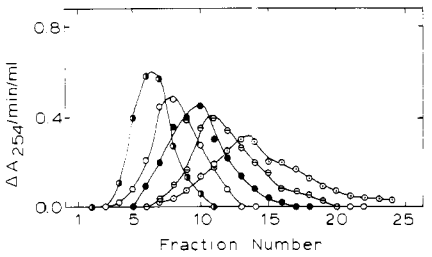


FIGURE 1: Competitive elution of CPB by D-Phe in the presence of 25 mM imidazole hydrochloride, pH 6.4; the column size was 4.0 × 0.9 cm, flow rate was 0.3 mL/min, and fractions of 1.6 mL were collected. [Gel-bound PheOH] was 5.4 mM by titration and amino acid analysis. [Soluble D-Phe]: (⊙) none added; (⊖) 2.12 mM; (●) 6.21 mM; (○) 10.5 mM; (◑) 21.6 mM.

term containing K_2 in eq 2 drops out and a linearized form (eq 3) may be obtained. The intercept and slope may then

$$\frac{1}{V_e - V_0} = \frac{K_3K_5}{(V_0 - V_m)[\overline{I\text{–}L}]}\frac{1}{[L_b]} + \frac{K_5}{(V_0 - V_m)[\overline{I\text{–}L}]} \quad (3)$$

be used to calculate K_3 and K_5. A similar approach has been used by Andrews et al. (1973) and Brinkworth et al. (1975).

The complexes described in the above schemes fall into two categories: mobile phase and stationary phase. The former arise from the interaction of soluble enzyme and added soluble ligand. Stationary-phase complexes contain the immobilized ligand ($\overline{I\text{–}L}$). In this case the term "stationary-phase" does *not* imply irreversibility. On the contrary, reversible binding does occur between $\overline{I\text{–}L}$ and the soluble protein or complex as depicted in the scheme for ligand induced retardation.

In our experiments, E refers to CBP; $\overline{I\text{–}L}$, to agarose–PheOH; L_a, to ligands such as D-Phe or *N*-acetyl-D-Phe; and L_b, to modifiers such as ϵAHx or βPP.

Results

Analysis of Affinity Absorbant. Agarose–PheOH, agarose–PheOME, and uncoupled Affi Gel 201 were analyzed by UV spectra, titration curves, and amino acid analysis. Titration data reveal that the titratable groups of Affi Gel 201 blocked by the reaction with PheOMe were recovered after saponification. Spectral data showed the characteristic Phe spectrum in both agarose–PheOMe and –PheOH. *In Particular, the spectrum after saponification indicated little or no loss of Phe.* The concentration of agarose-bound PheOH in μmol/mL was calculated as 8.7 by spectral analysis, 5.6 by titration analysis, and 5.3 by amino acid analysis.

Chromatography of CPB on Agarose–PheOH, Agarose–PheOMe, and Uncoupled Affi Gel 201. In the presence of 25 mM imidazole, pH 6.4, CPB was retarded by 5.5 bed volumes on agarose–PheOH. Under the same conditions, neither uncoupled Affi Gel 201 that contains free carboxyl groups nor agarose–PheOMe retarded the enzyme.

In the experiments just described the concentration of dialyzed CPB was 1.9×10^{-5} M. When the enzyme solution was diluted tenfold and chromatographed over agarose–PheOH, the elution profile was the same as that for the undiluted protein. The remaining experiments described in this paper utilized 2×10^{-5} M CPB.

Competitive Elution. Carboxypeptidase B, retarded by agarose–PheOH in the presence of 25 mM imidazole at pH 6.4, was competitively eluted by D-Phe (2–20 mM) and *N*-acetyl-D-Phe (1–18 mM) but not by 20 mM Gly. The effect of varying concentrations of D-Phe on the competitive elution of CPB is shown in Figure 1. When the data obtained with D-Phe and *N*-acetyl-D-Phe were plotted according to the equation of Dunn & Chaiken (1974), straight lines were

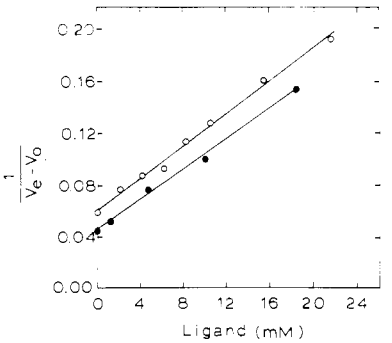


FIGURE 2: Plot of $1/V_e - V_0$ vs. concentration of soluble D-Phe and *N*-acetyl-D-Phe. The data in Figure 2 were plotted according to the equation for competitive elution (eq 1). (○—○) D-Phe; (●—●) *N*-acetyl-D-Phe.

Table I: Dissociation Constants for Carboxypeptidase B·Ligand Complexes from Competitive Elution Data[a]

ligand	K_D (mM)	
	K_1	K_2
D-Phe[b]	9.8	1.3
N-acetyl-D-Phe[c]	7.8	1.6

[a] Buffer: 25 mM imidazole, pH 6.4. [b] Column: 4.0 × 0.9 cm; flow rate = 0.3 mL/min; [I–Phe] = 5.4 mM. [c] Column: 6.5 × 0.9 cm; flow rate = 0.2 mL/min; [$\overline{I\text{–Phe}}$] = 6.7 mM.

obtained (Figure 2) showing that, under the conditions of the experiment, competitive elution was operative. The dissociation constants for the mobile-phase binary complexes (K_1), CPB·D-Phe and CPB·*N*-acetyl-D-Phe, and the stationary-phase complex (K_2), $\overline{\text{CPB·I-D-Phe}}$, were calculated from the slope and intercept values of eq 1 and are shown in Table I. Consideration of possible uncertainties in the parameters of eq 1 suggests error limits of ±20% for these values. The enzyme binds to soluble *N*-acetyl-D-Phe tighter than to soluble D-Phe by a factor of 1.3, but the stationary-phase CPB·agarose–PheOH complex is tighter than the soluble species by a factor of 7.5.

Ligand-Induced Retardation. The retardation of CPB on agarose–PheOH in 25 mM imidazole, pH 6.4, was increased by fivefold in the presence of 2 mM ϵAHx. Similarly in the presence of 1 mM βPP the elution volume increased twofold. By using the K_2 value obtained from competitive elution data and assuming the K_3 for CPB·ϵAHx is 1.5 mM and K_3 for CPB·βPP is 0.36 mM (Zisapel & Sokolovsky, 1972), eq 2 was used to determine K_5 at the single concentration of ϵAHx and βPP in the imidazole buffer. Our calculations showed that K_5 for $\overline{\text{CPB·I–Phe·ϵAHx}}$ is equal to 0.2 mM and K_5 for $\overline{\text{CPB·I–Phe·βPP}}$ is equal to 0.5 mM in 25 mM imidazole at pH 6.4.

The diffuse nature of the elution peak in the presence of ϵAHx and βPP as well as the extended length of time required for the experiment made an in depth study of retardation effects cumbersome. A buffer system was therefore sought which would enable us to carry out the chromatography in a convenient and reliable manner. The observations of Akanuma et al. (1971) led us to study ligand-induced retardation in the presence of phosphate buffer. These authors demonstrated that bovine CPB was retarded on a column of immobilized D-PheOH only in the presence of low concentrations of ϵAHx. In agreement with their observations, we found that porcine CPB was retarded by agarose–PheOH in the presence of phosphate buffer only if low concentrations of soluble ϵAHx were present in the eluting buffer. In addition we found that βPP retarded the enzyme under the same conditions. The CPB

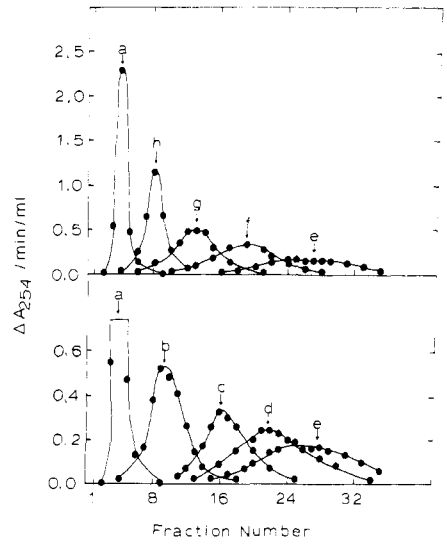


FIGURE 3: Effect of ϵAHx on the elution profile of CPB on agarose–PheOH in 10 mM phosphate, pH 7.2. A 6.5 × 0.9 cm column was used with a flow rate of 0.2 mL/min, and fractions of 1.6 mL were collected. [ϵAHx]: (a) none; (b) 0.2 mM; (c) 0.5 mM; (d) 1.0 mM; (e) 2.0 mM; (f) 5.0 mM; (g) 10 mM; (h) 20 mM.

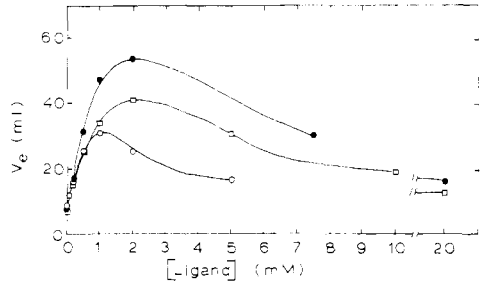


FIGURE 4: Concentration dependence of the effect of ϵAHx (●, pH 6.4; □, pH 7.2) and βPP (○, pH 6.4) on the elution profile of CPB on agarose–PheOH.

retarded in the presence of 2 mM ϵAHx was competitively eluted by 16 mM D-Phe such that the elution volume was approximately one-half that observed with ϵAHx alone. Neither 2 mM Gly, 2 mM D-Phe, 2 mM D-Arg, 0.5–2.0 mM *N*-acetyl-L-Arg nor 2 or 20 mM L-Lys induced the retardation of CPB on agarose–PheOH in 25 mM phosphate buffer at pH 6.4.

The effect of varying concentrations of ϵAHx on the elution profile of CPB on agarose–PheOH is shown in Figure 3. Similar results were obtained when βPP was used instead of ϵAHx. Cumulative data from three sets of experiments expressed as elution volume (V_e) vs. ligand concentration have been plotted in Figure 4. In each case a point of maximum retardation occurred after which the enzyme was eluted with increasing concentrations of ϵAHx or βPP. For ϵAHx this concentration was 2 mM and for βPP it was 1 mM.

The determination of the dissociation constant for each ternary complex used that portion of the elution volume–ligand concentration curve which showed retardation, i.e., the ascending portion of the curve in Figure 4. As a first approximation, eq 3 was used to extract K_3 (mobile phase binary complex) and K_5 (stationary phase ternary complex). The value for K_5 was further refined by using a program developed for eq 2 and assuming K_2 = 1 M which is a lower limit since binding is not observed in the absence of ϵAHx. A shift in the elution profile of less than 1 mL could be detected which would imply a K_2 of about 100 mM. The dissociation constants thus obtained are given in Table II.

The effect of pH and buffer composition on the ϵAHx

Table II: Dissociation Constants for Carboxypeptidase B·Ligand Complexes from Ligand Induced Retardation Data[a]

ligand	K_D (mM)	
	K_3	K_5
ϵAHx[b]	1.29	0.51
ϵAHx[c]	1.76	0.32
βPP[c]	0.65	0.98

[a] Concentration of immobilized Phe = 6.7 mM. Column: 6.5 × 0.9 cm. Flow rate = 0.2 mL/min. [b] 10 mM phosphate, pH 7.2. [c] 25 mM phosphate, pH 6.4.

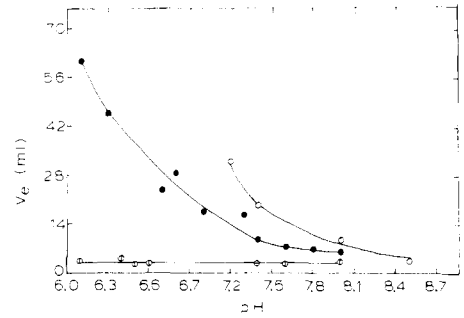


FIGURE 5: The pH profile of the binding of CPB to agarose–PheOH in the presence of 2 mM ϵAHx (●, 25 mM phosphate; ○, 25 mM Tris) and in the absence of ϵAHx (Φ, 25 mM phosphate; Θ, 25 mM Tris). A column of 3.6 × 0.9 cm was employed with [I-Phe] equal to 5.4 mM and a flow rate of 0.2 mL/min.

induced retardation of CPB on agarose–PheOH was investigated with phosphate (pK = 7.2) and Tris (pK = 8.1) buffers over a pH range of 6.1–8.5. In both cases the elution volume decreased as the pH increased (Figure 5). In the absence of ϵAHx, no retardation was observed from pH 6.1 to 8.0.

The conductivity, in mmhos, of the various solutions ranged from 3.8 (pH 8.0) to 1.8 (pH 6.1) for phosphate buffer and from 1.0 (pH 8.5) to 2.4 (pH 7.2) for Tris buffer. In order to control for differences in ionic strength at pH 7.4 where the binding in Tris was two times that in phosphate, sufficient NaCl was added to the former such that the final conductivity was the same as that of phosphate at pH 7.4. Under these conditions, the elution volume in Tris at pH 7.4 (final [NaCl] = 15 mM) changed from 19.7 to 15.3 mL. The elution volume in phosphate at pH 7.4 was 9.7 mL. Similar results were obtained with Tes at pH 7.4 except that, in the presence of this buffer with 2 mM ϵAHx, the binding of CPB to agarose–PheOH was 2.7 times that in the presence of phosphate.

The effect of 2 mM ϵAHx in 25 mM phosphate, pH 6.3, on the chromatographic profile of CPB on Affi Gel 201 was investigated to determine if the gel-bound Phe was necessary for the observed binding. Under these conditions, the elution volume of CPB on agarose–PheOH was 45.7 mL, while on Affi Gel 201 the enzyme eluted at 4.4 mL.

Discussion

The synthesis of agarose–PheOH from Affi Gel 201 was based on published procedures (Cuatrecasas, 1970; Tomino & Paigen, 1970; Nishikawa & Bailon, 1975a) except for the base hydrolysis of agarose–PheOMe. The recovery of the ultraviolet spectrum after base hydrolysis shows that this treatment did not result in scission of any bonds other than the COOMe. The uncertainty of the extinction coefficient of Phe when immobilized or in viscous solvents renders quantification of gel-bound Phe by UV spectroscopy unreliable. The measurement is, however, necessary to monitor the presence of the amino acid during the various synthetic steps.

The agreement between amino acid analysis and titration data shows that the conversion of the methyl ester to the free

acid was quantitative. For purposes of quantification of gel-bound Phe, the titrametric procedure is the most important because, as we have shown, only the immobilized free acid retarded CPB; gel-bound PheOMe groups were inert in this system. An accurate determination of gel-bound Phe is important because this number is required to obtain the dissociation constants for the stationary-phase enzyme·ligand species.[2]

Our results show that the interaction between agarose–PheOH and CPB is biospecific and strongly suggest that the enzyme binds to the affinity column through a D-Phe binding site. The retardation data agree with the observed hydrolyses of C-terminal Phe peptides (Wintersberger et al., 1962; Moore & Benoiton, 1972; Zisapel et al., 1973). In addition the inability of agarose–PheOMe to retard CPB is consistent with the known specificity of the enzyme for a free terminal carboxylate. The need for Phe on the column was shown by the lack of binding of CPB to Affi Gel 201 which contains free carboxyl groups but lacks the aromatic moiety.

Furthermore the binding of CPB to agarose–PheOH was diminished by the presence of soluble D-Phe or *N*-acetyl-D-Phe, whereas binding was unaffected by the presence of soluble Gly. Dependence of the elution volume on soluble D-Phe concentration follows the prediction of eq 1 for competitive elution. Such behavior is indicative of CPB binding through a site specific for immobilized D-Phe (O'Carra, 1974).

The enhanced retardation by low concentrations of soluble ϵAHx or βPP is consistent with effects of these modifiers that have been observed in affinity chromatographic (Akanuma et al., 1971) and kinetic studies (Moore & Benoiton, 1975). In the absence of immobilized D-Phe, no retardation was observed in the presence of ϵAHx. These observations further support the conclusion that the interaction between CPB and agarose–PheOH is a biospecific one.

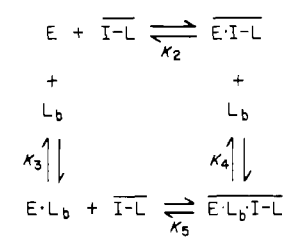
The greater affinity of CPB for the immobilized ligand (a situation also encountered with the RNase analogue) [Taylor & Chaiken, 1977] may be due to (a) interaction of the enzyme with the spacer arm and/or agarose matrix or (b) the effect of amino substitution. The first explanation is probably correct because the affinity of CPB for *N*-acetyl-D-Phe is only 1.3 times as great as that for the unsubstituted amino acid and is insufficient to account for the tighter binding of the protein to agarose–D-PheOH.

The structure of the spacer arm can dramatically influence the binding of the proteins to affinity columns (O'Carra et al., 1974; Nishikawa & Bailon, 1975b). In the case of CPB, however, such interactions have only mildly altered the binding characteristics of this protein to the immobilized ligand. Other workers, using different immobilized D-Phe derivatives, also found that CPB was retarded in the presence of imidazole at pH 6.5 (Uren, 1971) and that retardation in the presence of phosphate buffer required the presence of ϵAHx (Akanuma et al., 1971).

Agarose–spacer arm–D-Phe as a total entity could be analogous to a peptide carboxypeptidase substrate because of its large size. Our demonstration of enhanced binding in the presence of ϵAHx and βPP, however, shows that the spacer arm–D-Phe combination was behaving as a dipeptide analogue because activation phenomena disappear with large peptides (Zisapel et al., 1973; Zisapel & Sokolovsky, 1975; Auld & Vallee, 1970).

[2] This number represents the total concentration of bound D-Phe. If the quantity of immobilized ligand available for interaction with CPB is less than the total, the resulting K will be smaller.

Scheme I



A basic assumption in the derivation of eq 1 for competitive elution phenomena (Dunn & Chaiken, 1974) is the presence of soluble enzyme at a concentration much lower than K_2 (see Nichol et al., 1974). Hence, the concentration of enzyme does not appear in the equation. We tested whether or not our experiments followed this approximation by diluting the enzyme tenfold and chromatographing it over agarose–PheOH in the imidazole buffer. The observation of the same elution volume as that observed with the undiluted CPB showed that our experiments were conducted under conditions specified by the above assumption. In addition, the competitive elution experiments show that the concentration of applied CPB is about 100-fold less than that of the measured K_2. Hence, under the conditions of our experiments, the equation of Nichol et al. (1974) reduces to that of Dunn & Chaiken (1974).

Observed K_m and K_i values for various blocked glycyl and alanyl peptides terminating in Phe or phenyl acetate range from 0.035 to 138 mM (Alter et al., 1977; Moore & Benoiton, 1975; Zisapel et al., 1973; Zisapel & Sokolovsky, 1975). Elkins-Kaufman & Neurath (1949) in their study on CPA reported a K_i for D-Phe of 2 mM, but similar studies have not been reported for CPB. Due to the sensitivity of the kinetic parameters to blocking groups and side-chain structure, a comparison between our mobile phase and stationary phase dissociation constants and published values is not possible.

The retardation of CPB on agarose–Phe induced by low concentration of ϵAHx or βPP suggests the presence of multiple binding sites on the enzyme and is diagnostic for ternary complex formation (Akanuma et al., 1971). The solid-phase $\overline{\text{CPB}\cdot\epsilon\text{AHx}\cdot\text{agarose–PheOH}}$ complex might be the result of binding of the soluble CPB·ϵAHx complex to immobilized D-Phe or the stabilizing effect of ϵAHx on the immobilized CPB·D-Phe complex as seen in Scheme I.

Although our experiments cannot distinguish between these alternatives operating alone or together, we chose to analyze the data assuming the prior formation of the soluble binary complex (cf. also Schecter & Zazepizki, 1971), because it enabled us to compare K_3 for the soluble binary complex with literature values. Our values obtained at pHs 6.4 and 7.2 (Table II) are in fair agreement with K_is for ϵAHx (1.5 mM) and βPP (0.36 mM) obtained from solution studies at pH 7.9 (Zisapel & Sokolovsky, 1972). On the other hand, they are larger than expected from the chromatographic data obtained with bovine CPB (Akanuma et al., 1971).

The different chromatographic behavior of CPB on agarose–D-PheOH in imidazole and phosphate buffers at pH 6.4 may be due to enhanced binding in the presence of imidazole or to an inhibition of binding by phosphate. We feel that the results obtained in the absence of ϵAHx and βPP cannot be explained solely by phosphate inhibition because 25 mM acetate at pH 6.4 also will not induce retardation of CPB (unpublished results). Wolff et al. (1962) have shown that, whereas phosphate is inhibitory toward hippuryl-L-Arg hydrolysis, acetate is not. The decreased retention of CPB in the presence of ϵAHx by using phosphate compared with Tris may be a reflection of an inhibitory effect. It is important to

keep in mind that the action of imidazole is not the same as that of ϵAHx or βPP, because we were unable to induce retardation in phosphate by the addition of 2 mM imidazole to the elutant.

The pH dependence of ϵAHx induced retardation (increased V_e with a decrease in pH) may be a reflection of dissociation of (a) the buffer, (b) agarose–PheOH, (c) a component of CPB, or (d) a component of the CPB–ϵAHx complex. We favor explanation c or d, because the data shown in Figure 5 do not show an inflection close to the pK_a of either phosphate (pK_a = 7.2) or Tris (pK_a = 8.1). Furthermore, since CPB is not retarded by coupled Affi Gel 201 in the presence of ϵAHx, dissociation of the matrix cannot be a contributing factor. Either c or d would lead to pH-dependent ϵAHx binding to CPB. Kinetic studies (Zisapel & Sokolovsky, 1972) have indicated a pH dependence of K_m for basic substrates.

The requirement that Phe be in the free acid form on the affinity matrix and the presence of an acidic function on the enzyme to facilitate binding may be analogous to the binding of BSA to agarose bound aromatic residues. Hofstee (1973) showed that the binding of bovine serum albumin to an immobilized aromatic moiety (ϵ-aminohexanoyltryptophan methyl ester) was increased at low pH, but binding was negligible on unsubstituted matrices. We have shown that, in the presence of ϵAHx, the binding of CPB to a different immobilized aromatic moiety (phenylalanine) was also increased at low pH and was negligible on the unsubstituted matrix.

The presence of multiple binding sites on CPB has been suggested from kinetic (Moore & Benoiton, 1975; Zisapel & Sokolovsky, 1975) and affinity chromatographic experiments (Akanuma et al., 1971; Ager & Haas, 1977).

Moore & Benoiton (1975) showed that a number of compounds activated the hydrolysis of both basic and nonbasic substrates. Furthermore, the activation of hydrolysis of the nonbasic peptide was reflected by a decrease in the apparent K_m, not an increase in k_{cat}. Zisapel & Sokolovsky (1972) demonstrated that the modes of inhibition by a number of inhibitors were dependent on the substrate. Furthermore, in a study on the inhibition of hydrolysis of one CPB substrate by another, the same authors found that K_i and K_m values were often different, indicating that more than one binding site was available (Zisapel & Sokolovsky, 1975). Multiple sites which were hypothesized by Vallee et al. (1968) for CPA have been experimentally detected by circular dichroism studies (Alter & Vallee, 1978). In the latter investigation, separate binding sites were observed for Phe and βPP; in our study different sites have been postulated for Phe and βPP on CPB.

The sites discussed above may represent either activating or inhibiting modes of binding (Vallee et al., 1968). The enhanced retardation probably represents the activation mode, while the competitive elution effect at higher ligand concentrations represents the inhibitory mode of binding (Vallee et al., 1968; Moore & Benoiton, 1975). Furthermore, we found that the maximum concentration of βPP required for enhanced retardation was about 1 mM, and Moore & Benoiton (1975) found that the same concentration was needed for maximum activation of hippurylphenylalanine hydrolysis.

In our studies, D-PheOH competitively eluted CPB from agarose–PheOH in the presence or absence of 2 mM ϵAHx and in each case reduced the elution volume by about one-half. The similarity in the effect of soluble D-PheOH suggests that CPB is binding to immobilized D-PheOH through the same site in the presence and absence of ϵAHx.

The differences in the effects of ϵAHx, which induces strong binding of CPB to agarose–PheOH, and D-Arg, Lys, and acetyl-L-Arg, which do not, may be due to (a) different orientations of the ligands on the same site of the enzyme or (b) the presence of different binding modes for the effectors. We favor the first suggestion because the data of Wolff et al. (1962) and Zisapel & Sokolovsky (1972) show that all four components are competitive inhibitors of hippurylarginine hydrolysis by CPB. We have obtained similar results. Thus, when ϵAHx binds to CPB, the site or sites for D-Phe are still available and as discussed above the affinity is increased. Lys, D-Arg, and acetyl-L-Arg, on the other hand, although bound to CPB at the same sites as ϵAHx (Wolff et al., 1962), are oriented in such a way that binding to agarose–D-PheOH is not stimulated.

Quantitative affinity chromatography has been used in the past to calculate dissociation constants for protein–ligand interactions. Competitive elution data have been used in the study of staphylococcal nuclease (Dunn & Chaiken, 1974), ribonuclease (Taylor & Chaiken, 1977), and chymotrypsin (Gilbert, 1978). Ligand-induced retardation data were used to calculate dissociation constants for galactosyltransferase (Andrews et al., 1973) and lactate dehydrogenase (Brinkworth et al., 1975). In the above studies, K_D's in the micromolar to millimolar range were found. Our affinity chromatographic investigation of CPB has demonstrated that a stationary phase dissociation constant (K_2) in the millimolar range is probably an upper limit to the value that will permit a significant binding to the affinity matrix. Likewise, a K_D of 10 mM is probably a maximum value that will permit the mobile phase dissociation (K_1) constant to be accurately determined. The mobile phase K_D's (K_3) determined from ligand-induced retardation studies are within the range found by Brinkworth et al. (1975) and Andrews et al. (1973). Significantly, the study of Andrews et al. (1973) illustrates that weakly binding effectors can be analyzed by quantitative affinity chromatography. We have taken the concept a step further and analyzed the ternary complex that exists in the stationary phase. Hence, quantitative affinity chromatography provides a means for directly measuring the multiple binding sites and should be applicable to investigations of proteins that possess allosteric or autosteric binding sites.

References

Ager, S. P., & Haas, M. (1977) *Anal. Biochem. 83*, 285–295.

Akanuma, H., Kasuga, A., Akanuma, T., & Yamasaki, M. (1971) *Biochem. Biophys. Res. Commun. 45*, 27–33.

Alter, G. M., & Vallee, B. L. (1978) *Biochemistry 17*, 2212–2218.

Alter, G. M., Leussing, D. L., Neurath, H., & Vallee, B. L. (1977) *Biochemistry 16*, 3663–3668.

Andrews, P., Kitchen, B. J., & Winzor, D. J. (1973) *Biochem. J. 135*, 897–900.

Auld, D. S., & Vallee, B. L. (1970) *Biochemistry 9*, 602–609.

Brinkworth, R. I., Masters, C. J., & Winzor, D. J. (1975) *Biochem. J. 151*, 631–636.

Chaiken, I. M., & Taylor, H. C. (1976) *J. Biol. Chem. 251*, 2044–2048.

Cuatrecasas, P. (1970) *J. Biol. Chem. 245*, 3059–3065.

Davies, R. C., Auld, D. S., & Vallee, B. L. (1968) *Biochem. Biophys. Res. Commun. 31*, 628–633.

Dunn, B. M., & Chaiken, I. M. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2382–2385.

Elkins-Kaufman, E., & Neurath, H. (1949) *J. Biol. Chem. 178*, 645–654.

Folk, J. E. (1971) *Enzymes, 3rd Ed. 3*, 57–79.

Gilbert, W. A. (1978) Ph.D. Thesis, University of Florida.

Goldman, R., Goldstein, L., & Katchalski, E. (1971) in *Biochemical Aspects of Reactions on Solid Supports* (Stark, G. R., Ed.) pp 1–78, Academic Press, New York.
Greenstein, J. P., & Winitz, M. (1961) in *Chemistry of the Amino Acids*, Vol. 2, p 1689, Wiley, New York.
Hartsuck, J. A., & Lipscomb, W. N. (1971) *Enzymes, 3rd Ed. 3*, 1–56.
Hofstee, B. H. J. (1973) *Anal. Biochem. 52*, 430–448.
Moore, G. J., & Benoiton, N. L. (1972) *Biochem. Biophys. Res. Commun. 47*, 581–587.
Moore, G. J., & Benoiton, N. L. (1975) *Can. J. Biochem. 53*, 747–757.
Nichol, L. W., Ogston, A. G., Winzor, D. J., & Sawyer, W. H. (1974) *Biochem. J. 143*, 435–443.
Nishikawa, A. H., & Bailon, P. (1975a) *Anal. Biochem. 64*, 268–275.
Nishikawa, A. H., & Bailon, P. (1975b) *Arch. Biochem. Biophys. 168*, 576–584.
O'Carra, P. (1974) in *FEBS Symposium on Industrial Aspects of Biochemistry* (Spencer, B., Ed.) pp 107–134.
O'Carra, P., Barry, S., & Griffin, T. (1974) *FEBS Lett. 43*, 169–175.
Schecter, I., & Zazepizki, E. (1971) *Eur. J. Biochem. 18*, 469–473.
Taylor, H. C., & Chaiken, I. M. (1977) *J. Biol. Chem. 252*, 6991–6994.
Titani, K., Ericcson, L. H., Walsh, K. A., & Neurath, H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1666–1670.
Tomino, S., & Paigen, K. (1970) in *The Lactose Operon* (Beckwith, J. R., & Zipser, D., Eds.) pp 233–249, Cold Spring Harbor, New York.
Uren, J. R. (1971) *Biochim. Biophys. Acta 236*, 67–73.
Vallee, B. L., Riordan, J. J., Bethune, J. L., Coombs, T. L., Auld, D. S., & Sokolovsky, M. (1968) *Biochemistry 7*, 3547–3556.
Wintersberger, E., Cox, D. J., & Neurath, H. (1962) *Biochemistry 1*, 1069–1078.
Wolff, E., Schirmer, E. W., & Folk, J. E. (1962) *J. Biol. Chem. 237*, 3094–3099.
Zisapel, N., & Sokolovsky, M. (1972) *Biochem. Biophys. Res. Commun. 46*, 357–363.
Zisapel, N., & Sokolovsky, M. (1975) *Eur. J. Biochem. 54*, 541–547.
Zisapel, N., Kurn-Abramowitz, N., & Sokolovsky, M. (1973) *Eur. J. Biochem. 35*, 507–511.

Potentiometric Titrations and Oxidation–Reduction Potentials of Manganese and Copper–Zinc Superoxide Dismutases†

Glen D. Lawrence and Donald T. Sawyer*

ABSTRACT: Bovine erythrocyte superoxide dismutase and two manganese-containing superoxide dismutases have been reduced by the indirect coulometric titration method with methylviologen as the mediator–titrant. On the basis of the titration data the manganese-containing superoxide dismutases contain 1 g-atom of metal per mol of enzyme (dimer). $E^{0\prime}$ = +0.31 V for the enzyme from *Escherichia coli* which exhibits a complicated pH dependence above neutral pH. The *Bacillus stearothermophilus* manganese-containing enzyme has an $E^{0\prime}$ = +0.26 V and ΔE_m/pH is 50 mV. Bovine erythrocyte superoxide dismutase exhibits anomalous behavior in the coulometric titration curves, which is indicative of two nonequivalent copper centers in the enzyme. Addition of $K_3Fe(CN)_6$ or K_2IrCl_6 to the enzyme solution, prior to coulometric titration, indicates that these anions bind preferentially to one of the copper centers.

Although superoxide dismutases have been the subject of numerous studies in the past several years, the details of the oxidation–reduction chemistry for the enzymatic process are not understood (Fee, 1977). There is general agreement that all three classes of the enzyme (Cu/Zn-, Mn-, and Fe-containing SOD)[1] have a common mechanistic pathway for the catalysis of the disproportionation of superoxide ion ($O_2^{-}\cdot$), as shown by eq 1 and 2. However, the thermodynamics and

$$E_A + O_2^{-}\cdot \rightarrow E_B + O_2 \quad (1)$$

$$E_B + O_2^{-}\cdot + 2H^+ \rightarrow E_A + H_2O_2 \quad (2)$$

mechanisms for the individual electron transfer steps of the enzymatic process have not been determined (Fee, 1977).

The X-ray crystal structure of bovine Cu/ZnSOD has been determined to 3-Å resolution (Richardson et al., 1975a,b). For this enzyme the catalytic cycle has been postulated from pulse radiolytic investigations to be a ping-pong type mechanism (Klug et al., 1972; Klug-Roth et al., 1973), with the copper moiety undergoing alternate oxidation and reduction by $O_2^{-}\cdot$. The mechanism is complicated by the fact that H_2O_2 (one of the products of the reaction) causes the Cu(II) sites of the enzyme to be reduced (Klug-Roth et al., 1973; Rotilio et al., 1973; Bray et al., 1974). This poses a dilemma if one considers the nearly diffusion controlled rate of $O_2^{-}\cdot$ reduction by the enzyme and the redox potentials for the Cu(II)/Cu(I) and $O_2^{-}\cdot/H_2O_2$ couples. Fee & DiCorleto (1973) have determined that the Cu(II)/Cu(I) redox potential ($E^{0\prime}$) of BESOD has a value of +0.42 V vs. NHE. The potential for the $O_2^{-}\cdot/H_2O_2$ couple has been estimated to be +0.87 V (Fee & Valentine, 1977), a value much too positive to effect the direct reduction of the Cu(II) in the enzyme by H_2O_2.

The results of a detailed kinetic investigation of bovine SOD

† From the Department of Chemistry, University of California, Riverside, California 92521. *Received July 17, 1978; revised manuscript received February 22, 1979.* Supported by the U.S. Public Health Service, National Institutes of Health, under Grant No. GM 22761.

[1] Abbreviations used: BESOD, bovine erythrocyte superoxide dismutase; MnSOD, manganese-containing superoxide dismutase; FeSOD, iron-containing superoxide dismutase; MV^{2+}, methylviologen dication; $MV^{+}\cdot$, methylviologen cation radical; EPR, electron paramagnetic resonance; cyt *c*, horse heart cytochrome *c*; DCIP, 2,6-dichlorophenolindophenol.

0006-2960/79/0418-3045$01.00/0