tion are different (Gregory *et al.*, 1972). In this connection, one may consider the comparison of Michaelis constants for lactate dehydrogenase action on pyruvate and fluoropyruvate. Eisman *et al.* (1965) and Lee *et al.* (1965) report that the Michaelis constants for pyruvate and fluoropyruvate as substrates of lactate dehydrogenase are almost identical. It is now known through nuclear magnetic resonance measurements (J. E. Meany and J. Spaulding, unpublished results) that in contrast to pyruvate, fluoropyruvate is better than 85% hydrated at 25°. If one assumes, in analogy to pyruvate itself, that unhydrated fluoropyruvate is the preferential substrate of lactate dehydrogenase; and accordingly applies the respective correction factors $(1 - \chi)$ to the corresponding Michaelis constants, it is found that instead of being nearly identical, the complex constant for fluoropyruvate is about ten times smaller than that for pyruvate itself.

References

Albery, W. J., Bell, R. P., and Powell, A. L. (1965), *Trans. Faraday Soc. 61*, 1194.

Eigen, M., Kustin, K., and Strehlow, H. (1962), *Z. Physik. Chem. 31*, 140.

Eisman, E. H., Lee, H. A., and Winer, A. D. (1965), *Biochemistry 4*, 606.

Florini, J. R., and Vestling, C. S. (1957), *Biochim. Biophys. Acta 25*, 575.

Fromm, H. J. (1965), *Biochim. Biophys. Acta 99*, 540.

Gold, C. V., Socrates, G., and Crampton, M. R. (1964), *J. Chem. Soc.*, 588.

Gregory, D., Goodman, P. A., and Meany, J. E. (1972), *Biochemistry 11*, 4472.

Griffin, J. H., and Criddle, R. S. (1970), *Biochemistry 9*, 1195.

Griffiths, V. S., and Socrates, G. (1967), *Trans. Faraday Soc. 63*, 673.

Hegazi, M., and Meany, J. E. (1972), *J. Phys. Chem. 76*, 3121.

Jaenicke, R., and Knof, S. (1968), *Eur. J. Biochem. 4*, 157.

Lee, H. A., Eisman, E. H., and Winer, A. D. (1965), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 24*, 667.

Meany, J. E. (1971), *J. Phys. Chem. 75*, 150.

Millar, D. B., Frattali, V., and Willick, G. E. (1969), *Biochemistry 8*, 2416.

Pocker, Y., and Meany, J. E. (1970), *J. Phys. Chem. 74*, 1486.

Pocker, Y., Meany, J. E., Mist, B. J., and Zadorojny, C. (1969), *J. Phys. Chem. 73*, 2879.

Schwert, G. W., and Winer, A. D. (1963), *Enzymes 7*, 127.

Strehlow, H. (1962), *Z. Electrochem. 66*, 3921.

Vennesland, B. (1956), *Discussions Faraday Soc. 20*, 240.

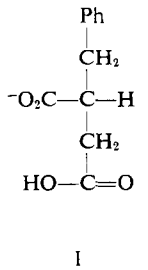
Winer, A. D., and Schwert, G. W. (1958), *J. Biol. Chem. 231*, 1065.

Binding of the By-Product Analog Benzylsuccinic Acid by Carboxypeptidase A†

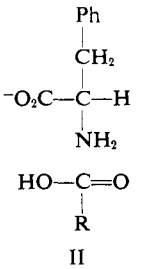
Larry D. Byers and Richard Wolfenden*

ABSTRACT: A variety of carboxylic and dicarboxylic acids were examined as inhibitors of bovine carboxypeptidase A. Of these, the most effective was 2(*R*)-benzyl-3-carboxypropionic acid (the L isomer of benzylsuccinic acid). This inhibitor appeared to be purely competitive with respect to hydrolysis of both esters and peptides, and exhibited $K_i = 4.5 (\pm 0.8) \times 10^{-7}$ M when assayed against a variety of substrates at pH 7.5 in 0.5 M NaCl at 25°. The stoichiometry, site, and pH dependence of binding were examined; the results suggest that a single molecule of a monoanionic species of L-benzylsuccinic acid is bound, and that this occurs at the active site. Binding is associated with detectable changes in ultraviolet spectra, resistance of the enzyme to various kinds of irreversible inactivation, an increase in enzyme solubility, and a change in the sites of enzyme derivatization with tetranitromethane. Kinetic analysis of enzyme inhibition by the products hippuric acid and L-phenylalanine suggests that binding of one of these products does not appreciably affect binding of the other. It is suggested that L-benzylsuccinate resembles the collected products of peptide hydrolysis, and is thus bound with an affinity resembling their combined affinity.

In a preliminary communication, we described the inhibition of bovine carboxypeptidase A by several carboxylic acids. In particular, it was noted that L-benzylsuccinic acid (I), which appears to resemble the collected substrates for the reverse reaction II, was an unusually potent inhibitor (Byers and Wolfenden, 1972).



I



II

In this paper, the structural requirements for tight binding of carboxylic acid inhibitors of carboxypeptidase A are considered in detail. The stoichiometry, site, and pH dependence

† From the Department of Biochemistry, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514. *Received November 28, 1972.* Supported by Research Grant GM-18325, Career Development Award AM-08560 (to R. W.), and Predoctoral Research Fellowship GM-49094 (to L. D. B.) from the National Institutes of Health.

of binding of L-benzylsuccinic acid have been explored, and an attempt has been made to examine some of the consequences of inhibitor binding for the physical and chemical properties of the protein.

Materials and Methods

Carboxypeptidase and Derivatives. Bovine carboxypeptidase A (Anson) was obtained from Worthington Biochemical Corp. Carboxypeptidase A (Cox) was prepared by the method of Cox *et al.* (1964). Zinc-free enzyme was prepared by the method of Coleman and Vallee (1961). In all studies with the apoenzyme, buffer solutions were extracted with a 3 mg % solution of dithizone in CCl_4. Cobalt carboxypeptidase A was prepared by the method of Latt and Vallee (1971). Acetyl-carboxypeptidase A was prepared with acetic anhydride by the method of Riordan and Vallee (1963).

Nitrocarboxypeptidase A was prepared by a modification of the method of Riordan *et al.* (1967a,b) by treatment of the native enzyme with a tenfold molar excess of tetranitromethane. The reaction was followed to completion spectrophotometrically at 350 nm, the λ_{max} of the nitroformate product (Sokolovsky *et al.*, 1966). After reaction with tetranitromethane for approximately 70 min (in 0.05 M Tris-HCl, pH 8.0, 2 M NaCl, room temperature) the protein was dialyzed against the same buffer at 4° for 15 hr. The number of nitro groups per protein molecule was estimated from the absorbance at 427 nm [ϵ_{426} for *N*-acetyl-3-nitrotyrosine (pK_a = 7.0) anion 4100 M^{-1} cm^{-1} (Riordan *et al.*, 1967b)] and at 381 nm (ϵ_{381} for this isosbestic point between protonated and unprotonated *N*-acetyl-3-nitrotyrosine 2200 M^{-1} cm^{-1} (Sokolovsky *et al.*, 1966)]. Results obtained at these two wavelengths were in good agreement with each other. Native protein concentrations were estimated from measurement of the absorbance at 278 nm, ϵ 6.42 $\times$ 10^4 M^{-1} cm^{-1} (Simpson *et al.*, 1963).

Enzyme Assays. Assays were performed as described previously (Byers and Wolfenden, 1972) for CGGP and BGPL.[1] Unless otherwise specified, assays were routinely carried out at pH 7.5 (0.025 M Tris-HCl–0.5 M NaCl, 25°). The following substrates were assayed by observation of the decrease in optical density at λ 230 nm: CGGT ($\Delta\epsilon_{230}$ −6290), CGGI ($\Delta\epsilon_{223}$ −336), *N*-formyl-L-phenylalanine ($\Delta\epsilon_{226}$ −589), and chloroacetyl-L-phenylalanine ($\Delta\epsilon_{233}$ −475).

pH Studies. pH values were measured at 25° with a Corning Model 12 pH meter. Assays at pH values varying from 5.4 to 6.0 were carried out in buffer solutions containing 0.025 M Mes, 0.5 M NaCl, and 10^{-4} M $ZnCl_2$. [Carboxypeptidase A is known to be inactivated by spontaneous loss of zinc at pH values below 5.5 (Vallee *et al.*, 1955)]. Assays at pH values varying from 6.4 to 9.0 were carried out in 0.025 M Tris-HCl (0.5 M NaCl). Some difficulty was encountered in assaying carboxypeptidase A at pH values above 10 due to inhibition by the buffer. Carbonate is a known inhibitor of the enzyme (Auld and Vallee, 1970b). However, the inhibition due to 0.02 M carbonate was small (less than 10%) and this buffer was employed accordingly. Triethylamine (0.025 M) was found to be an unacceptable buffer due to catalysis of substrate hydrolysis. Under all assay conditions used the substrate was found to be stable for at least 20 min.

Difference Spectra. Difference spectra were observed with a Bausch and Lomb Spectronic 505 spectrophotometer equipped with a scale expander such that a full recorder deflection corresponded to 0.1 OD unit. Samples were placed in tandem cuvets with a total optical path of 1.0 cm. Protein solutions were placed in one compartment. A base line was obtained by recording the spectrum with identical cuvets containing the unmixed solutions in both sample and reference beams. The difference spectrum was obtained by mixing the sample cuvet and recording the spectrum. All changes were observed to be rapid, yielding identical spectra throughout a 50-min period. Finally, the contents of the cuvet in the reference beam were mixed and a second base line was obtained in close agreement with the original base line.

Synthetic Procedures. D,L-Benzylsuccinic acid (2-benzyl-3-carboxypropionic acid) was prepared as described earlier (Byers and Wolfenden, 1972), or obtained commercially from Burdick and Jackson Co. and recrystallized from water.

L-Benzylsuccinic acid (I) (2(*R*)-benzyl-3-carboxypropionic acid) was prepared by resolution of the racemic acid either with α-chymotrypsin as described earlier (Byers and Wolfenden, 1972), or by crystallization with optically active α-methylbenzylamine (Aldrich Co.). Resolution with α-methylbenzylamine proved to be the method of choice since no esterification was required and larger quantities of the acid could be resolved. In a typical run D,L-benzylsuccinic acid (10.5 g) was dissolved in ethyl acetate (150 ml) followed by the addition, with stirring, of L(−)-α-methylbenzylamine (12.1 g). A white solid formed and was dissolved by adjusting the volume to 600 ml (with 50% ethyl acetate–50% methanol). Crystals formed on standing overnight in the cold (4°). The methylbenzylamine salt of the acid was removed by filtration and recrystallized four times from 60% ethyl acetate–40% methanol. After final recrystallization the salt was dissolved in 2 N H_2SO_4 and extracted with ether. The ether layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The resulting white solid was crystallized from water to yield 3 g of a compound with mp 161–163°[2] [lit. mp for L-benzylsuccinic acid 159–161° (Cohen and Milovanovic, 1968)] and $[\alpha]_D^{24}$ +27.2° (c 2.9, ethyl acetate) [lit. $[\alpha]_D^{20}$ +27° (Cohen and Milovanovic, 1968)].

2-*n*-Butyl-2-benzylmalonic acid (III) was prepared by saponification of diethyl 2-*n*-butyl-2-benzylmalonate [prepared by the method of Ukita *et al.* (1951)]. The diethyl ester [45 g, bp 165–170° (8 mm), lit. bp 135–139° (0.4 mm) (Ukita *et al.*, 1951)] was suspended in 55 ml of water containing 52 g of KOH and heated under reflux for 19 hr. The solution was cooled and added to 85 ml of concentrated HCl. This acidic aqueous solution was then extracted with ether. The ether layer was evaporated leaving a viscous liquid which solidified on standing. The solid was recrystallized from ethanol yielding rhombic crystals with melting point 103–105° (93% yield).

2-Butyl-3-phenylpropionic acid (IV) (2-benzylhexanoic acid) was prepared by decarboxylation of III. Compound III (34 g) was heated to 130° for 30 min to yield a yellowish liquid. The liquid was distilled and the fraction boiling at 158–162° (7 mm) was collected in 82% yield [lit. bp 127–130° (0.3 mm) (Ukita *et al.*, 1951)].

L-2-Benzylhexanoic acid (V) and D-2-benzylhexanoic acid (VI) were prepared by resolution of IV with α-methylbenzylamine. Compound IV (13.6 g) was added to 88 ml of ethyl

[1] Abbreviations used are: BAEE, *N*-benzoyl-L-arginine ethyl ester; BGPL, *N*-benzoylglycyl-L-phenyllactic acid; Cbz, carbobenzyloxy; CGGI, *N*-carbobenzyloxyglycylglycyl-L-isoleucine; CGGP, *N*-carbobenzyloxyglycylglycyl-L-phenylalanine; CGGT, *N*-carbobenzyloxyglycylglycyl-L-tryptophan; CGP, *N*-carbobenzyloxyglycl-L-phenylalanine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; TAME, *p*-toluenesulfonyl-L-arginine methyl ester.

[2] All melting points are uncorrected.

acetate containing 7.4 g of L(−)-α-methylbenzylamine. A white precipitate formed and was dissolved by bringing the volume to 380 ml with ethyl acetate. After standing 1 day crystals began to form. The crystals were collected by filtration and redissolved in ethyl acetate. Four recrystallizations yielded the L(−)-α-methylbenzylamine salt of V, with mp 125–126°. The free acid was obtained by dissolving the salt in 2 N H_2SO_4 and extracting with ether. Evaporation of the ether yielded a liquid with $[\alpha]_D^{20}$ +23° (ethyl acetate). Compound VI was obtained by treating the original mother liquor with D(+)-α-methylbenzylamine. The free acid showed the expected negative rotation at the sodium D wavelength. Assignment of absolute configuration was based on comparison of the rotation at the sodium D wavelength with that of L-2-methyl-3-phenylpropionic acid (Schrecker, 1957).

2-Allyl-2-benzylmalonic acid (VII) was prepared by a malonic ester condensation of diethyl allylmalonate with benzyl bromide in a method analogous to the preparation of III, to yield a product (58%) with mp 116–118° [lit. mp 113–115.5° (Arnold *et al.*, 1953)].

2-Benzyl-4-pentenoic acid (VIII) (2-allyl-3-phenylpropionic acid) was prepared by heating VII to 135°, yielding a product with bp 155–158° (3 mm) [lit. bp 144–146° (1.7 mm) (Arnold *et al.*, 1953)].

2-Benzyl-2-methylmalonic acid (IX) was prepared by a malonic ester condensation similar to the procedure of DeTar and Weis (1957) except that methyl iodide was used to alkylate diethyl benzylmalonate. The yield of diethyl methylbenzylmalonate was 69%. Saponification yielded a solid, which was recrystallized from benzene–hexane with mp 137–139° [lit. mp 138–139.5° (DeTar and Weis, 1957)].

2-Methyl-3-phenylpropionic acid (X) was prepared by thermal decarboxylation (at 160°) of IX. The resulting solid was crystallized from ethanol and yielded a solid with mp 37–39° [lit. mp 36.5–37.5° (DeTar and Weis, 1957)]. The racemic acid was resolved with α-methylbenzylamine by the method of Schrecker (1957) to yield L-2-methyl-3-phenylpropionic acid (XI) [bp 154–156° (4 mm); lit. bp 112° (0.25 mm) (Schrecker, 1957)] and D-2-methyl-3-phenylpropionic acid (XII) [bp 160–161° (5 mm); lit. bp 111° (0.2 mm) (Schrecker, 1957)].

2-Carboxymethyl-2-benzylmalonic acid (XIII) was prepared by the method of Cohen and Milovanovic (1968) and, after recrystallization from water, gave a melting point, with decomposition, of 176° [lit. mp 173° dec (Cohen and Milovanovic, 1968; Horii *et al.*, 1961)].

N-Benzoylaminoacetaldehyde (XIV) was prepared by the method of Fischer (1893). The aldehyde was not isolated but was prepared *in situ* by hydrolysis of the diethyl acetal of XIV [prepared by the method of Fischer (1893)].

N-Formyl-L-phenylalanine (XV) was prepared by the method of Fruton and Clark (1934), yielding a solid with mp 166–167° (lit. mp 167°).

N-Methanesulfonyl-L-phenylalanine (XVI) was prepared by the method of Cornish-Bowden and Knowles (1969) and found to have mp 106–107° (lit. mp 106–107°).

2-Cyano-3-phenylpropionic acid (XVII) was prepared by the method of Cordier (1945) and found to have mp 101–102° (lit. mp 100–101°).

Methyl hippurate (XVIII) was prepared by the method of Hammond and Gutfreund (1959) and found to have mp 80–82° (lit. mp 80–81.5°).

Hippurylamide (IX) was prepared from XVIII by the method of Hammond and Gutfreund (1959) and found to have mp 182–184° (lit. mp 182–184°).

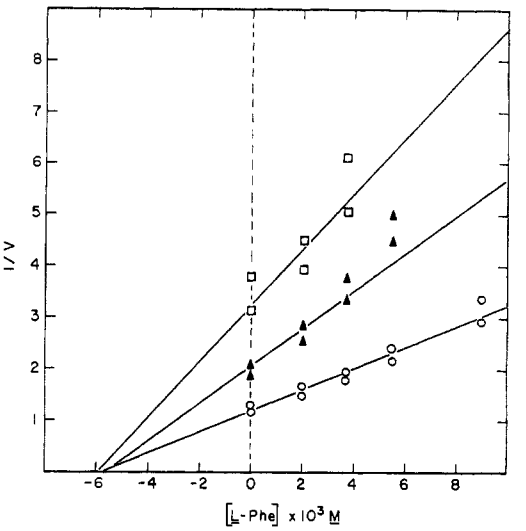


FIGURE 1: Yonetani–Theorell plot of the rate of hydrolysis of benzoylglycyl-L-β-phenyllactic acid catalyzed by carboxypeptidase A as a function of L-phenylalanine concentration at different levels of hippuric acid (benzoylglycine). The substrate concentration is 0.545 mM (pH 7.5, 0.02 M Tris-HCl–0.5 M NaCl, 25°). The velocities were observed in the absence of hippuric acid (○) and in the presence of 0.545 mM hippuric acid (▲) and 1.09 mM hippuric acid (□). The velocities are in arbitrary units.

2-(2-Carboxyethane)-3-phenylpropionic acid (XX) (D,L-2-benzylglutaric acid) was prepared by the method of Ansell and Hey (1950) and found to have mp 77–78° (lit. mp 76.5–78°).

L-2-Benzyl-3-carboethoxypropionic acid (XXI) was prepared by the method of Cohen and Milovanovic (1968) and found to have mp 41–42° (lit. mp 41–42°).

D,L-Dimethyl benzylsuccinate (XXII) and the half ester, L-2-benzyl-3-carbomethoxypropionic acid (XXIII) were prepared as described earlier (Byers and Wolfenden, 1972).

N-Acetylglycylglycine (XXIV) was prepared by a method similar to that described by Bergmann *et al.* (1929).

Other Materials. Other enzymes and reagents were used as obtained from commercial sources.

Results

Reversible Inhibitors. Structural requirements for inhibitor binding were examined by determining the inhibition constants of various carboxylic acids with respect to the hydrolysis of the substrate carbobenzyloxyglycylglycyl-L-phenylalanine (CGGP) from double reciprocal plots of the dependence of velocity on substrate concentration in the presence and absence of inhibitor as previously described (Byers and Wolfenden, 1972). Inhibition appeared to be competitive[3] in all cases where discrete K_i values could be determined (Table I).

Independent Binding of Product Inhibitors. In order to determine the possible mutual influence of binding of the two products of hydrolysis of a typical substrate, the rate of hydrolysis of benzoylglycyl-L-β-phenyllactic acid (BGPL) was measured as a function of L-phenylalanine concentration at different levels of hippuric acid (Figure 1). The lines appear to intersect

[3] Inhibitors were considered competitive when the lines in a double reciprocal plot (in the presence and absence of inhibitor) appeared, within experimental error, to intersect at the ordinate. A more direct approach, based on enzyme interactions with inhibitors and fluorescent substrates, was reported (Auld *et al.*, 1972) after submission of this paper.

TABLE I: Inhibitors of Carboxypeptidase A.[a]

β-Phenylpropionic Acids, $PhCH_2CHRCO_2H$ R	10^4K_i (M)
H	1.0[b]
D,L-CH_3 (X)	9.8
D-CH_3 (XII)	8.6
L-CH_3 (XI)	12.8
D,L-CH_2=$CHCH_2$ (VIII)	28
D,L-$CH_3CH_2CH_2CH_2$ (IV)	13
D-$CH_3CH_2CH_2CH_2$ (V)	10
L-$CH_3CH_2CH_2CH_2$ (VI)	17
D,L-NC (XVII)	100
D-^+H_3N	19[c]
L-^+H_3N	55[d]
D,L-HO	2.5
L-HO	1.3[e]
O=	>200[f]
L-CH_3C(=O)NH	15
L-CH_3SOONH (XVI)	6.7
L-HC(=O)NH (XV)	22
L-Cbz-NH	6

Benzylmalonic Acids, $PhCH_2CR(COOH)_2$ R	10^5K_i (M)
H	6[g]
CH_3 (IX)	16
CH_2=$CHCH_2$ (VII)	5
$CH_3CH_2CH_2CH_2$ (III)	2.2
HO_2CCH_2 (XIII)	1
H (monoethyl ester)	300

Acyl Portion Products and Analogs Compound	K_i (M)
N-CbzGly	3×10^{-2}[h]
N-CbzGlyGly	1.7×10^{-2}[i]
N-Acetylglycine	5×10^{-2}
N-Acetylglycylglycine	2×10^{-2}
Hippuric acid	8×10^{-4}[j]
Methyl hippurate	7×10^{-4}
Hippurylamide	2×10^{-4}
N-Benzoylaminoacetaldaldehyde	5×10^{-4}
N-Benzoylaminoacetaldaldehyde, Diethyl acetal	$>5 \times 10^{-2}$

Dicarboxylic Acids Compound	K_i (M)
Succinic acid	5×10^{-4}[k]
Maleic acid	3×10^{-4}[k]
Fumaric acid	30×10^{-4}[k]
D,L-Phenylsuccinic acid	2×10^{-4}
D,L-Benzylglutaric acid (XX)	5×10^{-6}
D,L-Dimethyl benzylsuccinate	$>10^{-2}$
D,L-Benzylsuccinic anhydride	$>5 \times 10^{-2}$[l]
L-2-Benzyl-3-carbethoxy-propionic acid	9.6×10^{-6}
L-2-Benzyl-3-carbomethoxy-propionic acid	6.3×10^{-6}
D,L-Benzylsuccinic acid	$1.1 (\pm 0.3) \times 10^{-6}$
D-Benzylsuccinic acid	3×10^{-6}
L-Benzylsuccinic acid	4.5×10^{-7}

[a] Assays carried out as described in text at pH 7.5 (0.25 M Tris-HCl–0.5 M NaCl, 25°) against the substrate CGGP. Maximum error in K_i is about 11%. [b] $K_i = 6.2 \times 10^{-5}$ M against CGP (Elkins-Kaufman and Neurath, 1949); $K_i = 1.52 \times 10^{-4}$ M against hippuryl-L-mandelate (Kaiser and Carson, 1965). [c] $K_i = 2 \times 10^{-3}$ M when measured against CGP (Elkins-Kaufman and Neurath, 1949). [d] $K_i = 5.57 \times 10^{-3}$ M when measured against CGP (Whitaker, 1966). [e] $K_i = 8.4 \times 10^{-5}$ M when measured against *O*-(*trans*-cinnamoyl)-L-β-phenyllactate (Kaiser and Kaiser, 1969). [f] A concentration of 10^{-2} M inhibitor was reported to give 19% inhibition against 0.025 M CGP at 37° (Geratz, 1965). [g] $K_i = 4 \times 10^{-3}$ M against CGP (Smith *et al.*, 1951). [h] Concentration necessary for 50% inhibition of 10^{-2} M hippuryl-D,L-β-phenyllactate is 0.15 M; this compound accelerates the rate of hydrolysis of CGP (Vallee *et al.*, 1968). [i] This compound also accelerates the rate of hydrolysis of hippuryl-D,L-β-phenyllactate (Vallee *et al.*, 1968). [j] This compound does not appear to inhibit the carboxypeptidase A catalyzed hydrolysis of CGP (Elkins-Kaufman and Neurath, 1948). [k] Estimated from the amount of inhibitor necessary for 50% inhibition. [l] Hydrolyzed spontaneously; K_i value extrapolated to zero time.

near the abscissa, suggesting (Yonetani and Theorell, 1964) that the presence of one inhibitor does not appreciably affect the binding of the other.

Site and Stoichiometry of Binding of L-*Benzylsuccinic Acid* (I). As we noted previously, L-benzylsuccinic acid was observed to be an inhibitor competitive with respect to the hydrolysis of BGPL, BGP, and CGGP (Byers and Wolfenden, 1972). This inhibitor has also been found to be competitive with respect to the hydrolysis of CGGT, CGGI, chloroacetyl-L-phenylalanine, and *N*-formyl-L-phenylalanine, with K_i values in all cases equal to 4.5 (±0.8) $\times 10^{-7}$ M at pH 7.5 and 25°. This value is slightly lower than the value originally reported (6×10^{-7} M) with a somewhat less pure preparation of the inhibitor. The rate of hydrolysis of CGGP was measured as a function of the concentration of L-benzylsuccinic acid at different levels of L-β-phenyllactic acid (Figure 2), a competitive inhibitor which is bound at a single locus at the active site (Hartsuck and Lipscomb, 1971). Parallel lines were obtained, indicating mutually exclusive binding. In order to determine whether L-benzylsuccinic acid is also bound at a single locus, a titration of enzyme activity was performed at pH 5.4, where the inhibitor is very tightly bound (see below). The results, plotted according to the procedure of Henderson (1972), indicate that carboxypeptidase appears, kinetically, to bind 1 mol of inhibitor under these conditions (Figure 3). Experiments at pH 7.5 yielded similar results. As noted previously (Byers and Wolfenden, 1972), L-benzylsuccinic acid, in very slight molar excess over the enzyme, afforded efficient protection against inactivation of carboxypeptidase by an irreversible inhibitor.

pH Dependence of Inhibitor Binding. Inhibition by L-benzylsuccinic acid appeared to be purely competitive through the pH range from 6.0 to 10.1. At lower pH values it was more difficult to demonstrate the nature of the inhibition since the enzyme is less active under these conditions and the affinity of the enzyme for the inhibitor is so high that "mutual deple-

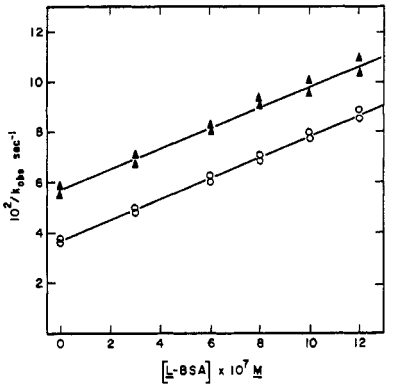


FIGURE 2: Yonetani–Theorell plot of the rate of hydrolysis of carbobenzoxyglycylglycyl-L-phenylalanine (8×10^{-4} M at pH 7.5 in 0.025 M Tris-HCl–0.5 M NaCl, at 25°) catalyzed by carboxypeptidase A as a function of L-benzylsuccinic acid in the absence of L-β-phenyllactic acid (○) and in the presence of 2×10^{-4} M L-β-phenyllactic acid (▲). The concentration of carboxypeptidase A is 10^{-8} M.

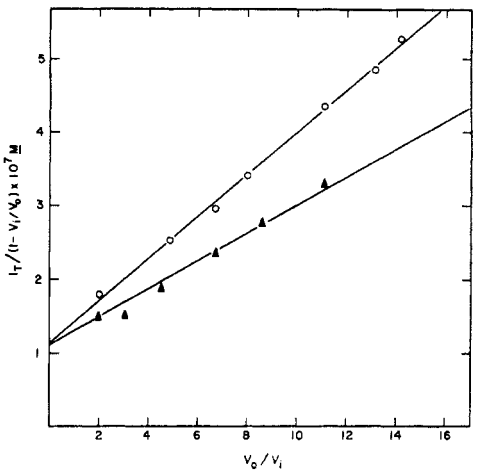
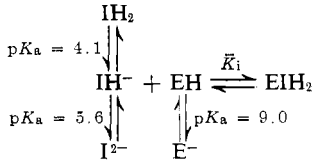


FIGURE 3: Effect of L-benzylsuccinic acid on the rate of the carboxypeptidase A catalyzed hydrolysis of CGGP at pH 5.4 (0.025 M Mes–0.5 M NaCl–10^{-4} M $ZnCl_2$, 25°). I_t is the total concentration of inhibitor present, v_i is the observed initial velocity in the presence of the inhibitor, and v_0 is the observed initial velocity in the absence of the inhibitor (Henderson, 1972). The assays were carried out in the presence of 1.16×10^{-7} M carboxypeptidase A and at substrate (CGGP) concentrations of 1.6 mM (○) and 0.75 mM (▲).

tion" (see Webb, 1963) could not be avoided. However, the Henderson plot (Figure 3) obtained by titration of enzyme activity at pH 5.4 showed an increase in slope with increasing substrate concentration, suggesting that inhibition was also competitive under these conditions (Henderson, 1972).

Figure 4 shows the K_i values observed for inhibition of the hydrolysis of CGGP by L-benzylsuccinic acid as a function of changing pH. Earlier studies indicate that the enzyme shows two kinetically significant pK_a values with this substrate. V_{max} falls off at and below apparent pK_a = 6.2, and K_m rises at and above apparent pK_a = 9 (Auld and Vallee, 1971; we have obtained a similar result). L-Benzylsuccinic acid has two ionizable groups, with apparent pK_a values of 4.1 and 5.6 under conditions of our assay (see also Weast, 1967). The *solid line* in Figure 4 is a theoretical curve drawn on the basis of the following assumed equilibria



where the value for the pH-independent dissociation constant $\bar{K}_i = 6.4 \times 10^{-9}$ M. The *broken line* is a theoretical curve based on the additional assumption that EH is protonated below pK_a = 6.2 to give a form of the enzyme (EH_2) which does not combine with IH^-. The solid line appears to give the better fit.

Protective Effects of L-Benzylsuccinic Acid on the Enzyme. ZINC REMOVAL. EDTA, at pH 7.5, is an effective inactivating agent of carboxypeptidase A, presumably due to removal of zinc. Carboxypeptidase A (3.2×10^{-7} M in 0.025 M Tris-HCl (pH 7.5)–0.5 M NaCl) was observed to be inactivated by 9.3×10^{-8} M EDTA (as measured by activity loss for the hydrolysis of CGGP) with a half-time of about 1 min. In the presence of 5.5×10^{-4} M L-benzylsuccinic acid the half-time for activity loss was increased to about 50 min. Similar results were obtained with the use of 1,10-phenanthroline as a zinc chelating agent.

PROTEOLYSIS. Autoproteolytic digestion of carboxypeptidase A (4.3×10^{-6} M) at 37° and pH 7.3 (in 0.25 M Tris-HCl–1 M NaCl) was found to be less than 5% after 150 min. Trypsin (1.67 mg/ml) at pH 7.0 slowly inactivated the enzyme at 37° (in 0.025 M Tris-HCl–1 M NaCl). About half of the enzymic activity (toward BGPL) was lost after 2 hr. With 5×10^{-6} M L-benzylsuccinic acid present (benzylsuccinic acid does not inhibit trypsin; see Table II) less than 10% of the original activity was lost in the same time period. Pronase (0.33 mg/ml) was found to inactivate carboxypeptidase A (6.6×10^{-7} M) at pH 8.0 (0.025 M Tris-HCl–1 M NaCl) and 37° with a half-time of 52 min. When 2×10^{-5} M L-benzylsuccinic acid was present the half-time for Pronase inactivation was greater than 850 min. Benzylsuccinic acid was found not to inhibit the BAEEase or TAMEase activity of Pronase (see Table II).

HEAT INACTIVATION. Petra and Neurath (1969) examined the heat inactivation of various species of carboxypeptidase A at 50° and found that the half-time for denaturation varied from about 5 to 50 min depending on the species of the enzyme (which varied in size and amino acid composition). In the present study, a heterogeneous carboxypeptidase A (Cox, 1964) preparation was employed. At 50° the half-time for heat inactivation of a 9.4×10^{-8} M solution of the enzyme (pH 7.2, 0.025 M Tris-HCl–0.5 M NaCl) was 40 min when assayed against BGPL. In the presence of 1×10^{-5} M L-benzylsuccinic acid, the half-time was increased to greater than 20 hr. With β-phenyllactic acid present (2.9×10^{-3} M) instead of L-benzylsuccinic acid, the half-time for inactivation was about 610 min.

Interaction of L-Benzylsuccinic Acid with Nitrated Carboxypeptidase A. Carboxypeptidase A was nitrated with tetranitromethane in the presence of L-benzylsuccinic acid [to yield nitrocarboxypeptidase A (+)] and in the absence of inhibitor [to yield nitrocarboxypeptidase A (−)] by the procedure described under Materials and Methods. When carboxypeptidase A (1.9×10^{-4} M) was allowed to react with tetranitromethane (2×10^{-3} M) at room temperature, pH 7.5 (in 0.03 M Tris-HCl–2 M NaCl), for 70 min in the presence of L-benzylsuccinic acid (3×10^{-4} M) a derivatized carboxypeptidase A was obtained containing 0.93 (±0.04) mol of NO_2/mol of carboxypeptidase A. In the absence of the inhibitor,

TABLE II: Enzymes Not Subject to Inhibition by L-Benzylsuccinic Acid (L-BSA).

Enzyme	pH	Substrate	[S] (mM)	[L-BSA] (M)[a]	Assay
α-Chymotrypsin	7.0	PNPA	1	5×10^{-5}	*b*
α-Chymotrypsin	7.6	ATEE	0.5	3×10^{-4}	*c*
Trypsin	8.0	BAEE	0.5	5×10^{-5}	*c*
Trypsin	8.1	TAME	3	1×10^{-4}	*d*
Papain	6.2	BAEE	0.5	1×10^{-4}	*c*
Pepsin	2.4	Hb	14 mg/ml	1×10^{-4}	*e*
Pronase	8.0	BAEE	0.5	1×10^{-4}	*c*
Pronase	8.1	TAME	3	1×10^{-4}	*d*

[a] The concentration of L-benzylsuccinic acid tested when no inhibition of the enzymic activity of various proteolytic enzymes was detected. Substrates are: *p*-nitrophenyl acetate (PNPA), *N*-acetyl-L-tyrosine ethyl ester (ATEE), *N*-benzoyl-L-arginine ethyl ester (BAEE), *p*-toluenesulfonyl-L-arginine methyl ester (TAME), and hemoglobin (Hb). [b] Hartley, B. S., and Kirby, B. A. (1954), *Biochem. J. 56*, 288. [c] Schwert, G. W., and Tanenaka, Y. (1955), *Biochim. Biophys. Acta 16*, 570. [d] Hummel, B. C. W. (1959), *Can. J. Biochem. Phys. 37*, 1393. [e] Anson, M. L. (1938), *J. Gen. Phys. 22*, 79.

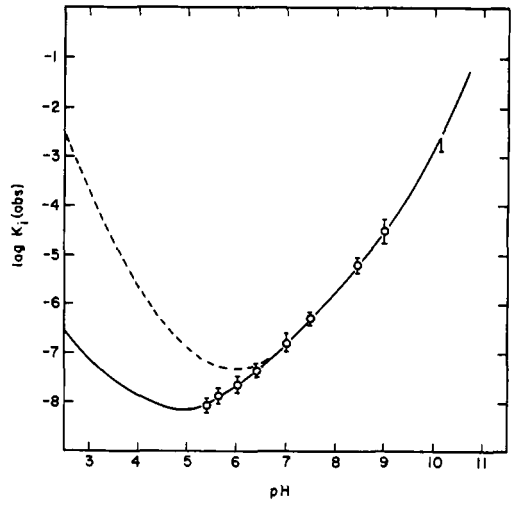


FIGURE 4: pH dependence of K_i of L-benzylsuccinic acid. Assays were performed at 25° in 0.5 M NaCl with carbobenzoxyglycylglycyl-L-phenylalanine as substrate. The solid line is the theoretical curve assuming the anionic forms of the inhibitor, IH^-, bind equally well to the two forms of the enzyme, EH_2 and EH^-. The dashed line is the theoretical curve assuming IH^- binds only to EH^-. K_i values in the pH range 5.6–9.0 were obtained from double reciprocal plots. The value at pH 5.4 was obtained from a Henderson plot (Figure 3). The value at pH 10.1 is a lower limit for K_i obtained at this pH.

under identical conditions, a derivatized enzyme containing 0.94 (±0.04) mol of NO_2/mol of carboxypeptidase A was obtained. Benzylsuccinic acid, thus, had virtually no effect on the extent of the reaction of the enzyme with tetranitromethane.

When difference spectra were compared (Figure 5) it became evident that the spectral properties were also different for the nitrocarboxypeptidases depending on whether benzylsuccinic acid had been present or absent when the native enzyme was nitrated. Figure 5 indicates the difference spectra for [nitrocarboxypeptidase A (+) + I mixed] *vs.* [nitrocarboxypeptidase A (−) + I unmixed] (dashed curve). When nitrocarboxypeptidase A (−) was examined in the same way a different perturbation spectrum was obtained (solid curve). Benzylsuccinic acid induces an observable change in the ultraviolet spectrum of nitrocarboxypeptidase A where the nitrotyrosine serves as a reporter group, but this change is different for the two species of nitrocarboxypeptidase A, suggesting a different environment of the nitro group. It has been observed that the 3-phenylpropionic acid decreases the absorbance of nitrocarboxypeptidase A at λ 428 nm and increases the absorbance around 350 nm (Sokolovsky *et al.*, 1966). Similar results were obtained on L-benzylsuccinic acid binding to nitrocarboxypeptidase A (−). However, on binding of the inhibitor to nitrocarboxypeptidase A (+) a decrease in absorbance was seen around 428 and 360 nm and an increase in absorbance was observed centered around 385 nm.

When carboxypeptidase A (2.0×10^{-4} M) was nitrated in the presence of L-benzylsuccinic acid (3.5×10^{-4} M) with a tenfold excess of tetranitromethane (at pH 7.5, 0.04 M Tris-HCl–1.9 M NaCl, room temperature) for 90 min a derivatized enzyme containing 1.1 mol of NO_2/mol of carboxypeptidase A was obtained. No further nitration was found to occur upon further treatment with another tenfold excess of tetranitromethane over protein in the presence of benzylsuccinic acid for 70 min. The protein was then dialyzed to remove the inhibitor and this nitrocarboxypeptidase A (+) preparation was again treated with a tenfold excess of tetranitromethane for 75 min. The resulting derivatized enzyme contained 2.4 mol of NO_2/mol of carboxypeptidase A indicating nitration of a second tyrosine residue in the absence of inhibitor. These results, along with the spectral studies described above, suggest that nitration of carboxypeptidase A in the presence of L-benzylsuccinic acid occurs at a different residue or residues than nitration in the absence of inhibitor. This is in contrast to the behavior of 3-phenylpropionic acid, which protects car-

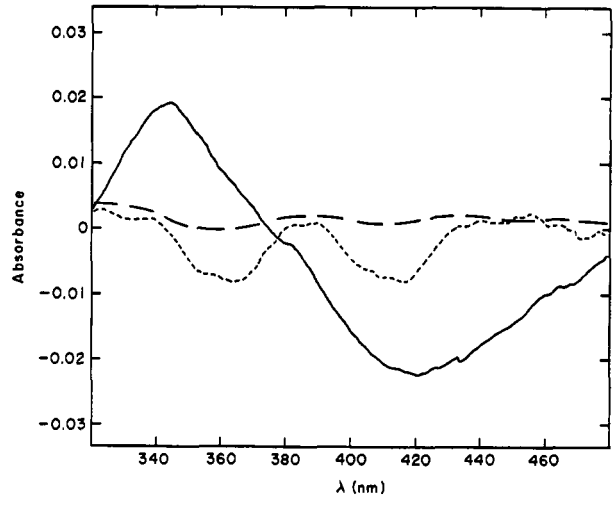


FIGURE 5: Difference spectra of nitrocarboxypeptidase A and L-benzylsuccinic acid. The nitrocarboxypeptidase A contained 0.93 ± 0.04 nitro group/molecule. The difference spectra were obtained following mixing of the enzyme (final concentration = 5×10^{-4} M). The solid curve is for the nitrocarboxypeptidase A (−) nitrated in the absence of L-benzylsuccinic acid and the dashed curve is for the nitrocarboxypeptidase A (+) nitrated in the presence of the inhibitor, a described in the text. The final pH is 7.5 (0.02 M Tris-HCl–0.4 M NaCl).

boxypeptidase A against nitration and yields enzyme that has unaltered catalytic properties (Riordan *et al.*, 1967b).

Other Physical Changes Induced by Benzylsuccinic Acid. L-Benzylsuccinic acid (1 mM) was found to increase the solubility of carboxypeptidase A (from 4 mg/ml, in 0.08 M NaCl (pH 7.5)–0.02 M Tris-HCl, room temperature) about fivefold. A similar effect of 3-phenylpropionic acid on the solubility of carboxypeptidase A has been observed (Petra, 1970). The rate at which the enzyme dissolves also appears to be increased in the presence of L-benzylsuccinic acid.

Experiments in collaboration with Drs. F. Quiocho and W. N. Lipscomb have shown that a 1 mM solution of the inhibitor leads to disordering of the crystal structure of both native and cross-linked carboxypeptidase A. No other inhibitor was found to disorder carboxypeptidase A crystals at such a low concentration.

L-Benzylsuccinic acid (5×10^{-4} M) induces an increase in the ultraviolet absorbance of a 2.1×10^{-5} M carboxypeptidase A solution (0.5 M NaCl (pH 7.5)–0.025 M Tris-HCl, 25°) at 298 nm. This increase is similar to that induced by 3-phenylpropionic acid, phenyllactic acid, D-phenylalanine, and D-tyrosine as reported by Fujioka and Imahori (1962) but of larger magnitude ($\Delta\epsilon_{298}$ 840 M^{-1} cm^{-1}) than that reported for the other inhibitors ($\Delta\epsilon_{298}$ values are all about 200 M^{-1} cm^{-1}). Preliminary experiments on the influence of racemic benzylsuccinic acid (2.5×10^{-5} M) on the optical rotory dispersion spectrum of carboxypeptidase A (8×10^{-6} M in 0.25 M Tris-HCl (pH 7.5)–10% LiCl, 25°) indicate that the inhibitor induces a change in the 260–400-nm region of the spectrum, similar to that caused by 3-phenylpropionic acid (Fujioka and Imahori, 1962).

Preliminary ultracentrifuge studies with the Rayleigh interference optical technique as described by Richards and Schachman (1959) indicate that a small increase in the sedimentation coefficient of carboxypeptidase A is induced by the addition of L-benzylsuccinic acid (8×10^{-4} M) to a solution of carboxypeptidase A (5 mg/ml in 2 M NaCl at pH 7.5, 20°, 0.025 M Tris-HCl). Under similar conditions, 3-phenylpropionic acid does not alter the sedimentation coefficient of carboxypeptidase A by more than the amount which would be expected simply from the increased effective molecular weight of the protein due to inhibitor binding (Kirschner and Schachman, 1971).

Importance of Zinc. L-Benzylsuccinic acid preincubated with zinc chloride is as effective an inhibitor of carboxypeptidase A as is L-benzylsuccinic acid with no exogenous zinc ions present. L-Benzylsuccinic acid is a reversible and time-independent inhibitor of carboxypeptidase A.

When 0.05 ml of an apocarboxypeptidase A preparation (1.5×10^{-5} M, containing less than 14% zinc as estimated by atomic absorption spectroscopy) was incubated with 0.05 ml of 3×10^{-5} M zinc-free L-benzylsuccinic acid for 1 min followed by incubation with 0.05 ml of zinc chloride (10^{-4} M) for 1 min and assayed for CGGP hydrolytic activity, the activity was lower, by about 45%, than when the addition of L-benzylsuccinic acid and zinc was reversed. Thus, benzylsuccinic acid appears to reduce the rate of reactivation of the apoenzyme by zinc.

L-Benzylsuccinic acid also inhibited the carboxypeptidase A like activity of Pronase from *Streptomyces griseus* K-1 which apparently requires Ca^{2+} (Narahashi and Yanagita, 1967; Narahashi *et al.*, 1968). This activity was measured by hydrolysis of BGPL (7×10^{-4} M) and CGGP (9×10^{-4} M) at pH 7.5 (in 0.025 M Tris-HCl–0.5 M NaCl). The amount of inhibitor necessary for 50% inhibition of the esterase activity was about 2×10^{-5} M. L-Benzylsuccinic acid appears to inhibit the CGGP hydrolytic activity of cobalt carboxypeptidase A (Auld and Vallee, 1970a) at pH 7.5 (0.025 M Tris-HCl–0.5 M NaCl and 10^{-4} M $CoCl_2$) with $K_i = 1.9 (\pm 0.7) \times 10^{-7}$ M.

Discussion

3-Phenylpropionic acid appears to be bound more tightly than any of its derivatives (Table I) and was, until recently, the most effective known reversible inhibitor of carboxypeptidase A (Hartsuck and Lipscomb, 1971). Under some conditions, 3-phenylpropionic acid shows mixed inhibition (Auld and Latt, 1971), and crystallographic (Steitz *et al.*, 1967) and spectral studies (Fretto and Strickland, 1971) have shown that it binds at two distinct loci in the active-site region. In contrast, D- and L-phenylalanine and L-β-phenyllactic acid have been shown to be bound at a single locus (Quiocho *et al.*, 1971).

The present findings establish that L-benzylsuccinic acid is bound at a single locus at the active site of carboxypeptidase A, and is an extremely effective reversible inhibitor, competitive against a variety of substrates. Inquiring as to the sources of its high affinity for the enzyme, we may first note that the presence of two carboxylic acid groups appears to be critical, and that the corresponding malonic and glutaric acid derivatives are considerably less effective.

We have previously noted that inhibition by benzylsuccinic acid is not due to inactivation by removal of the essential zinc atom, since the inhibitor does not lead to progressive inactivation of the enzyme. Since two carboxylic acid groups are required, inhibition might appear to be due to chelation of the metal at the active site. Several findings suggest that this is not the case. First, benzylsuccinic acid appears to be bound as the monoanion (Figure 4), whereas the dianion might be expected to be more effective as a chelating agent. It must be noted, however, that the observed results might be obtained if the dianion were to displace hydroxide ion as a zinc ligand, so that chelation is not definitely excluded by this result. Second, the half-esters of benzylsuccinic acid are rather effective inhibitors, although the second carboxylate group is blocked. Unpublished results of our own and of E. T. Kaiser (personal communication) show that L-2-benzyl-3-carbomethoxypropionic acid and L-2-benzyl-3-carbethoxypropionic acid are not detectably hydrolyzed by carboxypeptidase A. The half-ethyl ester XXI continues to inhibit after many recrystallizations, so that the observed inhibition is not due to contamination with the free dicarboxylic acid. Finally, the influence of benzylsuccinic acid on the recovery of activity of the apoenzyme, when the latter is incubated in the presence of zinc, suggests that benzylsuccinic acid may combine with the metal-free enzyme. This remains to be proven by direct binding experiments. It should be added that succinic acid derivatives appear to be rather poor chelating agents for free zinc (Martell, 1964). For these various reasons, we incline to the view that benzylsuccinic acid may supply one, but is unlikely to supply two, of the ligands to zinc in the enzyme–inhibitor complex.

In evaluating the binding of benzylsuccinic acid, we may begin by noting that this compares favorably with the affinity of hydrolytic substrates. Benzylsuccinic acid is 3000 times more tightly bound than the "good" peptide substrate CGGP, for which K_m appears (Auld and Vallee, 1970b) to be a true binding constant. It is of interest to compare the K_i of L-benzylsuccinic acid with the combined K_i values of hydrolytic products, substrates for the reverse (peptide-forming) reaction. We find (Table I) that hippuric acid is bound with K_i =

8×10^{-4} M, and L-phenylalanine is bound with $K_i = 5.5 \times 10^{-3}$ M at pH 7.5. It is significant that the Yonetani–Theorell plot (Figure 1) indicated that one of these inhibitors did not affect the binding of the other; otherwise, it might have been considered that hippuric acid might be bound in both the acyl and leaving group portions of the active site, the latter corresponding to nonproductive binding. The anionic form of phenylalanine is presumably the immediate product of hippuryl-L-phenylalanine hydrolysis and the reactant in the reverse reaction; correcting for the effects of ionization ($pK_a = 9.1$ for the phenylalanine zwitterion under the conditions of these experiments), the K_i value for the phenylalanine anion may be estimated as approximately 10^{-5} M. Making the same kind of correction for hippuric acid ($pK_a = 3.6$ under the conditions of these experiments) one obtains a virtual K_i value for the conjugate acid of hippuric acid (a presumed product of hydrolysis and effective reactant in the reverse, peptide forming, reaction) of approximately 10^{-7} M. The product of these virtual K_i values is on the order of 10^{-12} M^2. To obtain the approximate dissociation constant which might be expected of a single inhibitory molecule, perfectly combining the binding properties of the substrates for the reverse reaction, a substantial downward correction of this number should be made for the *cratic* contribution (Gurney, 1953). Even without this correction, this number is lower than the value (6.4×10^{-9} M) calculated for the monoanion of L-benzylsuccinic acid.

It may be noted that benzylsuccinic acid lacks some of the binding determinants of the acyl portion of the substrate and that hippuric acid (arbitrarily chosen for this comparison) has an unusually high affinity among the possible acyl products of the action of the enzyme. It might be argued that L-benzylsuccinic acid shares some structural characteristics of chemical intermediates in peptide hydrolysis. However, in view of the numerical considerations noted above, we prefer to adopt the more conservative view that benzylsuccinic acid is a "byproduct analog," combining characteristics of the two products of ester or peptide hydrolysis in a single molecular species (Byers and Wolfenden, 1972).

If this view is correct, it must nevertheless be noted that the forms of the products chosen for comparison involve unusual states of ionization which are thermodynamically unstable at neutrality. They might thus be represented as points removed some distance from the origin along the reaction coordinate for the reverse (peptide bond forming) reaction. It is of interest that the structure of L-benzylsuccinic acid (I) suggests a possible orientation of the incipient products (substrates for the reverse reaction) on the enzyme II. This particular orientation is not *required* by (nor is it necessarily inconsistent with) mechanisms involving the formation of an acyl-enzyme type intermediate. Products after they were fully formed by such a mechanism might in principle lie on the enzyme in almost any relative orientation. If, on the other hand, carboxypeptidase A acts as a general base catalyst for water attack on the bound substrate, the immediate products of hydrolysis would be expected to lie in a relative configuration very like that proposed for the inhibitor. To this limited extent, the present findings may tend to support the latter mechanism without definitely excluding the first. It may be noted that the distances separating the carbon atoms α to the two carboxyl groups of benzylsuccinic acid (I) are rather closer than they would be expected to be in the fully developed products (II), but that extension of this distance as in benzylglutartic acid is less detrimental to binding than is further contraction, as in benzylmalonic acid.

It will be of interest to determine the protein origins of the high affinity of benzylsuccinic acid for carboxypeptidase A. The present results show that inhibitor binding results in detectable changes in ultraviolet spectra, sedimentation coefficient, resistance to various forms of inactivation, and sites of nitration. These results suggest that inhibitor binding may be accompanied by a change in the structure of the enzyme. Further studies will be required to determine whether a substantial change in protein conformation is involved, and whether corresponding changes occur during catalysis.

Acknowledgment

We wish to thank Dr. Ben Evans for advice and assistance with synthetic procedures and analysis, Dr. Curtis Harper for help with atomic absorption studies, Miss Barbara Bamman for performing the ultracentrifuge studies, and Drs. W. N. Lipscomb and F. A. Quiocho for the opportunity of inspecting a model of carboxypeptidase A.

References

Ansell, M. F., and Hey, D. H. (1950), *J. Chem. Soc.*, 1683.
Arnold, R. T., Campos, M., and Lindsay, K. L. (1953), *J. Amer. Chem. Soc. 75*, 1044.
Auld, D. S., and Latt, S. A. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30* (II), 1078.
Auld, D. S., Latt, S. A., and Vallee, B. L. (1972), *Biochemistry 11*, 4994.
Auld, D. S., and Vallee, B. L. (1970a), *Biochemistry 9*, 602.
Auld, D. S., and Vallee, B. L. (1970b), *Biochemistry 9*, 4352.
Auld, D. S., and Vallee, B. L. (1971), *Biochemistry 10*, 2892.
Bergmann, M., DuVineaud, V., and Zervas, L. (1929), *Chem. Ber. 62*, 1909.
Byers, L. D., and Wolfenden, R. (1972), *J. Biol. Chem. 247*, 606.
Cohen, S., and Milovanovic, A. (1968), *J. Amer. Chem. Soc. 90*, 3495.
Coleman, J. E., and Vallee, B. L. (1961), *J. Biol. Chem. 236*, 2244.
Cordier, P. (1945), *C. R. Acad. Sci. 220*, 177.
Cornish-Bowden, A. J., and Knowles, J. R. (1969), *Biochem. J. 113*, 363.
Cox, D. J., Bovard, F. C., Bargetzi, J. P., Walsh, K. A., and Neurath, H. (1964), *Biochemistry 3*, 44.
DeTar, D. F., and Weis, C. (1957), *J. Amer. Chem. Soc. 79*, 3045.
Elkins-Kaufman, E., and Neurath, H. (1948), *J. Biol. Chem. 175*, 893.
Elkins-Kaufman, E., and Neurath, H. (1949), *J. Biol. Chem. 178*, 645.
Geratz, J. D. (1965), *Arch. Biochem. Biophys. 111*, 134.
Gurney, R. W. (1953), Ionic Processes in Solution, New York, N. Y., McGraw-Hill, p 89.
Fischer, E. (1893), *Chem. Ber. 26*, 464.
Fretto, L., and Strickland, E. H. (1971), *Biochim. Biophys. Acta 235*, 473.
Fruton, J. S., and Clarke, H. T. (1934), *J. Biol. Chem. 106*, 667.
Fujioka, H., and Imahori, K. (1962), *J. Biol. Chem. 237*, 2804.
Hammond, B. R., and Gutfreund, H. (1959), *Biochem. J. 72*, 349.
Hartsuck, J. A., and Lipscomb, W. N. (1971), *Enzymes, 3rd Ed. 3*, 1.
Henderson, P. J. F. (1972), *Biochem. J. 127*, 321.

Horii, Z., Saki, T., Tamura, Y., and Tanaka, K. (1961), *Chem. Pharm. Bull. 9*, 442.
Kaiser, B. L., and Kaiser, E. T. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 36.
Kaiser, E. T., and Carson, F. W. (1965), *Biochem. Biophys. Res. Commun. 18*, 457.
Kirschner, M. W., and Schachman, H. K. (1971), *Biochemistry 10*, 1900.
Latt, S. A., and Vallee, B. L. (1971), *Biochemistry 10*, 4253.
Martell, A. E., Ed. (1964), *Chem. Soc., Spec. Publ. No. 17*, 409.
Narahashi, Y., Shibuya, K., and Yanagita, M. (1968), *J. Biochem. (Tokyo) 64*, 427.
Narahashi, Y., and Yanagita, M. (1967), *J. Biochem. (Tokyo) 62*, 633.
Petra, P. H. (1970), *Methods Enzymol. 19*, 460.
Quiocho, F. A., Bethge, P. H., Lipscomb, W. N., Studebaker, J. F., Brown, R. D., and Koeing, S. H. (1971), *Cold Spring Harbor Symp. Quant. Biol. 35*, 561.
Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63*, 1578.
Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1967a), *Biochemistry 6*, 358.
Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1967b), *Biochemistry 6*, 3609.
Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 1460.
Schrecker, A. W. (1957), *J. Org. Chem. 22*, 33.
Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.
Smith, E. L., Lumry, R., and Polglase, W. (1951), *J. Phys. Chem. 55*, 125.
Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3582.
Streitz, T. A., Ludwig, M. L., Quiocho, F. A., and Lipscomb, W. N. (1967), *J. Biol. Chem. 242*, 4662.
Ukita, C., Tamemasa, O., and Motomatsu, H. (1951), *J. Pharm. Soc. 71*, 1044.
Vallee, B. L., Riordan, J. F., Bethune, J. L., Coombs, T. L., Auld, D. S., and Sokolovsky, M. (1968), *Biochemistry 7*, 3547.
Vallee, B. L., Rupley, J. A., Coombs, T. L., and Neurath, H. (1955), *J. Biol. Chem. 217*, 253.
Weast, R. C., Ed. (1967), Handbook of Tables for Organic Compound Identification, 3rd ed, Cleveland, Ohio, Chemical Rubber Publishing Co.
Webb, J. L. (1963), Enzyme and Metabolic Inhibitors, Vol. I, New York, N. Y., Academic Press.
Whitaker, J. R. (1966), *Biochem. Biophys. Res. Commun. 22*, 6.
Wolfenden, R. (1972), *Accounts Chem. Res. 5*, 10.
Yonetani, T. and Theorell, H. T. (1964), *Arch. Biochem. Biophys. 106*, 243.

Ultraviolet Difference Spectroscopic Studies of the Binding of Ligands to Rabbit Muscle Aldolase†

Alvin L. Crowder, III, Robert Barker,* and Charles A. Swenson

ABSTRACT: The binding of D-arabinitol 1,5-diphosphate, 1,5-pentanediol diphosphate, and inorganic phosphate to rabbit muscle aldolase (EC 4.1.2.7) has been investigated by ultraviolet difference spectroscopy. Binding of D-arabinitol 1,5-diphosphate, a potent inhibitor, causes a change in the environment of a tryptophan residue. The hydroxyl groups of the ligand are necessary for perturbation of the tryptophan residue. By ultraviolet difference spectroscopic studies of the binding of arabinitol 1,5-diphosphate in the presence of ethylene glycol the environmental change was shown to arise from a conformational adjustment in the enzyme which increases the exposure of tryptophan to solvent.

Binding of the competitive inhibitor D-arabinitol 1,5-diphosphate (Ara-P_2[1]) to rabbit muscle aldolase (EC 4.1.2.7) has been shown to produce changes in the environment of the tyrosyl and tryptophyl residues as measured by ultraviolet difference spectroscopy (Lehrer and Barker, 1971). Certain competitive inhibitors have been shown to quench the fluorescence of rabbit muscle aldolase (Rose and O'Connell, 1969). Similar results were obtained with rat muscle aldolase (Suh and Barker, 1971) and the quenching was shown to depend upon the presence and orientation of hydroxyl and/or carboxyl groups in the inhibitor. In addition the presence of hydroxyl groups can increase the binding constant by an order of magnitude as can the orientation of hydroxyl groups in polyhydroxylic derivatives (Hartman and Barker, 1965; Suh and Barker, 1971). In the present paper two aspects of ligand binding are examined by ultraviolet difference spectroscopy. First, the relationship between the quenching of fluorescence and the generation of a difference spectrum when a ligand is bound to aldolase was examined. Second, the cause of the spectral changes that occur on binding was sought using difference spectroscopy in the presence of the solvent perturbant ethylene glycol.

Experimental Section

Materials. Rabbit muscle aldolase was prepared from adult New Zealand rabbits (6–8 lb) by the method of Taylor *et al.* (1948) as modified by Lehrer and Barker (1971).

† From the Department of Biochemistry, University of Iowa, Iowa City, Iowa 52240. *Received November 10, 1972.* Supported in part by a Public Health Service Grant (HD 02618) and a Public Health Service Research Career Development award to C. A. Swenson (GM 42,384) from the Institute of General Medical Sciences.

[1] Abbreviations used are: Ara-P_2, D-arabinitol 1,5-diphosphate; Pen-P_2, 1,5-pentanediol diphosphate; P_i, inorganic orthophosphate.