Structure of an actively exchanging complex between carboxypeptidase A and a substrate analogue

(enzyme catalysis/proteases/protein crystallography)

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ABSTRACT   An x-ray diffraction study at 2.8 Å resolution
has yielded the structure of a complex between bovine car-
boxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.17.1)
and (−)-2-benzyl-3-p-methoxybenzoylpropionic acid. This sub-
strate is an analogue of N-(p-methoxy)-benzoylphenylalanine,
in which the amide NH is replaced by CH_2. T. Sugimoto and E.
T. Kaiser [(1979) *J. Am. Chem. Soc.* 101, 3946–3951] have shown
that this complex catalyzes stereospecific exchange of that
proton of the CH_2 group which is in the R configuration. Our
structure of this complex supports the model proposed by Su-
gimoto and Kaiser and is very similar to the productive peptide
binding mode suggested by Lipscomb et al. [Lipscomb, W. N.,
Hartsuck, J. A., Reeke, G. N., Quiocho, F. A., Bethge, P. A.,
Ludwig, M. L., Steitz, T. A., Muirhead, H. & Coppola, J. C. (1968)
Brookhaven Symp. Biol. 21, 24–90]. The proposed roles of glu-
tamic acid 270 in the proton exchange and the interaction of
zinc with the carbonyl group of the substrate are consistent with
the observed structure.

The wide range of kinetic phenomena observed in the hy-
drolysis of peptide and ester substrates by bovine carboxy-
peptidase A (CPase A) (peptidyl-L-amino-acid hydrolase, EC
3.4.17.1) suggests either a variety of binding modes or a variety
of rate-determining steps, or both. On the basis of crystallo-
graphic studies, at high resolution, of interactions of small
peptides with this enzyme, Lipscomb and coworkers (1–4) have
proposed productive, activating, and inhibitory modes of
binding of peptide substrates. Two closely related, but distinct,
mechanisms for the initial stages of peptide bond cleavage are
consistent with these structural investigations and with most
of the kinetic and chemical modification studies (1–4). The
contribution of the structural studies so far is to invoke Glu 270
or Glu 270 · · · H_2O as the nucleophile attacking the substrate's
carbonyl carbon, Tyr 248 (or H_2O) as the proton donor to the
amide nitrogen, and Zn^{2+} to polarize the substrate's carbonyl
bond by binding to oxygen and activating the carbon for this
initial nucleophilic atack. However, there are complexities and
subtle differences among the various plausible steps in the in-
termediate stages (4) of these mechanisms, and thus formidable
difficulties remain in elucidating the detailed mechanism of
catalysis by CPase A.

Sugimoto and Kaiser (5, 6) have recently approached this
problem by studying the enolization by CPase A of a ketonic
substrate, in which hydrogen–deuterium exchange is stereo-
specifically promoted in one hydrogen of the CH_2 group ad-
jacent to the CO group. The compound selected for this study,
(−)-2-benzyl-3-p-methoxybenzoylpropionic acid (BMBP), is
an analogue of N-(p-methoxy)-benzoylphenylalanine in which
the NH group of the scissile peptide bond has been replaced by
CH_2 in the ketonic substrate (Fig. 1). As a result, there is no
cleavage reaction. The stereospecific exchange at the CH_2 oc-
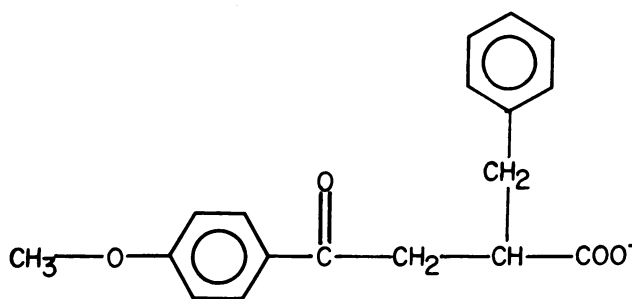

FIG. 1.   Structure of BMBP.

curs at the proton of R configuration, with retention of con-
figuration at the methylene carbon. From the known absolute
configuration of BMBP and of the active site of CPase A, and
from the known binding mode of the dipeptide glycyltyrosine
to CPase A (1), Sugimoto and Kaiser implicated Glu 270 in the
proton exchange reaction.

Because Glu 270 is possibly involved in both peptide hy-
drolysis and in this enolization–exchange process, we have
undertaken an x-ray diffraction study of the complex of this
ketonic substrate with CPase A, as described below. Our results
support the model for binding of this ketonic substrate as pro-
posed by Sugimoto and Kaiser and stress the similarities and
differences between the BMBP interaction and the interaction
that leads to the productive mode for peptide substrates as
proposed by Lipscomb and coworkers.

MATERIALS AND METHODS

CPase A (Cox) was purchased from Sigma and used without
further purification. Crystals of CPase A with the same cell
dimensions as used in the previous x-ray structural work (1)
were grown from 0.2 M LiCl/20 mM Veronal buffer, pH 7.5.
Gradual additions of BMBP, a generous gift of E. T. Kaiser, over
a period of 48 hr, brought the final concentration of BMBP in
the crystal soaking buffer to 2 mM. Crosslinking of the crystals
with glutaraldehyde (7) eliminated a serious cracking problem
at even low concentrations (0.1 mM) of BMBP.

A 2.8-Å data set was collected from a single crystal on a
Syntex $P2_1$ diffractometer at 4°C. The decay in the intensities
of four check reflections was less than 15% at the end of data
collection. The data were corrected for absorption (8) and Lo-
rentz-polarization effects. A difference electron density map
of the CPase A–BMBP complex minus the native CPase A
structure was calculated by using the Fourier coefficients
$(F_{complex} − F_{native}) \exp(i\phi_{mir})$, where $F_{complex}$ is the structure
factor of the complex, F_{native} is the native enzyme structure

factor, and ϕ_{mir} is the multiple isomorphous replacement phase determined for the native structure.

RESULTS AND DISCUSSION

The difference electron density map clearly shows one molecule of BMBP binding to the active site of CPase A. Comparison of the peak heights in this map to peak heights in previously studied complexes of peptides and CPase A indicates an occupancy for BMBP of approximately 30% under the soaking conditions used (1). Sections of the difference electron density map and stereoviews of the active site region are shown in Figs. 2 and 3. Because the resolution of this study is 2.8 Å, uncertainties of as much as 1.0 Å may be present in the location of specific atoms. The difference electron density, map, however, reveals unambiguously the major points of interaction between this ketonic substrate and CPase A.

The molecule binds in a manner consistent with the productive binding mode proposed for peptide substrates. The benzyl group of BMBP, identical to a phenylalanine side chain, extends into the main hydrophobic pocket of CPase A. Arg 145 interacts with the carboxylate group of the substrate, as shown by difference electron density, which shows conformational shifts in this residue. The methylene group, site of the stereospecific hydrogen exchange, is located next to the carboxylate ion of Glu 270, with the adjacent carbonyl oxygen of the substrate directed towards the zinc. Movement of the zinc by approximately 1.0 Å towards the keto group implies a direct interaction between the carbonyl oxygen and metal. Due to the expulsion of a zinc-liganded water, however, there is no continuous density between the carbonyl carbon and metal. Several other water molecules are displaced from the active site region upon binding of BMBP, including sites near Glu 270, Tyr 198, and a second nonligand site near the zinc. The *p*-methoxy-

benzoyl group extends into the hydrophobic region of Tyr 198 and Phe 279, with the methoxy group sufficiently close to Tyr 198 for a direct interaction.

Comparison of the complexes of BMBP and glycyltyrosine with CPase A reveals very similar binding modes for the two molecules. Within experimental error, the location of the carboxyl, carbonyl, and phenyl groups coincide. Protein conformational changes in these ligand–enzyme structures are similar: the large motion of the Tyr 248 side chain and the smaller movements of the zinc and the Arg 145 side chain are observed in both complexes. However, the few dissimilarities are probably significant for the proposed hydrolytic mechanisms of peptides. The amino group of glycyltyrosine interacts with Glu 270 through an intervening water molecule. Lipscomb *et al.* (1, 4) have proposed that this interaction is responsible for the low rate of hydrolysis of dipeptides with an unblocked amino group by CPase A. Substitution of a *p*-methoxybenzyl group for the amino group of glycyltyrosine eliminates this interaction, and indeed, there is no difference density attributable to Glu 270. An additional, but subtle, difference between the two complexes occurs in the carboxylate region of the substrate. In the glycyltyrosine complex, Arg 145 shows strong difference electron density, with relatively weak difference density corresponding to Tyr 248. The opposite situation exists with BMBP: by far the dominant difference electron density arises from the large motion of Tyr 248. In addition, because BMBP lacks an amide nitrogen, the hydroxyl group of Tyr 248 apparently forms a hydrogen bond with the carboxylate group. Such an interaction clearly favors the un-ionized state of Tyr 248 at pH 7.5. The observed shift in position of Tyr 248 between the two complexes, from the amide nitrogen to the carboxylate group, may displace Arg 145 less in the BMBP complex, thus accounting for the relatively weak difference electron density corresponding to this residue.
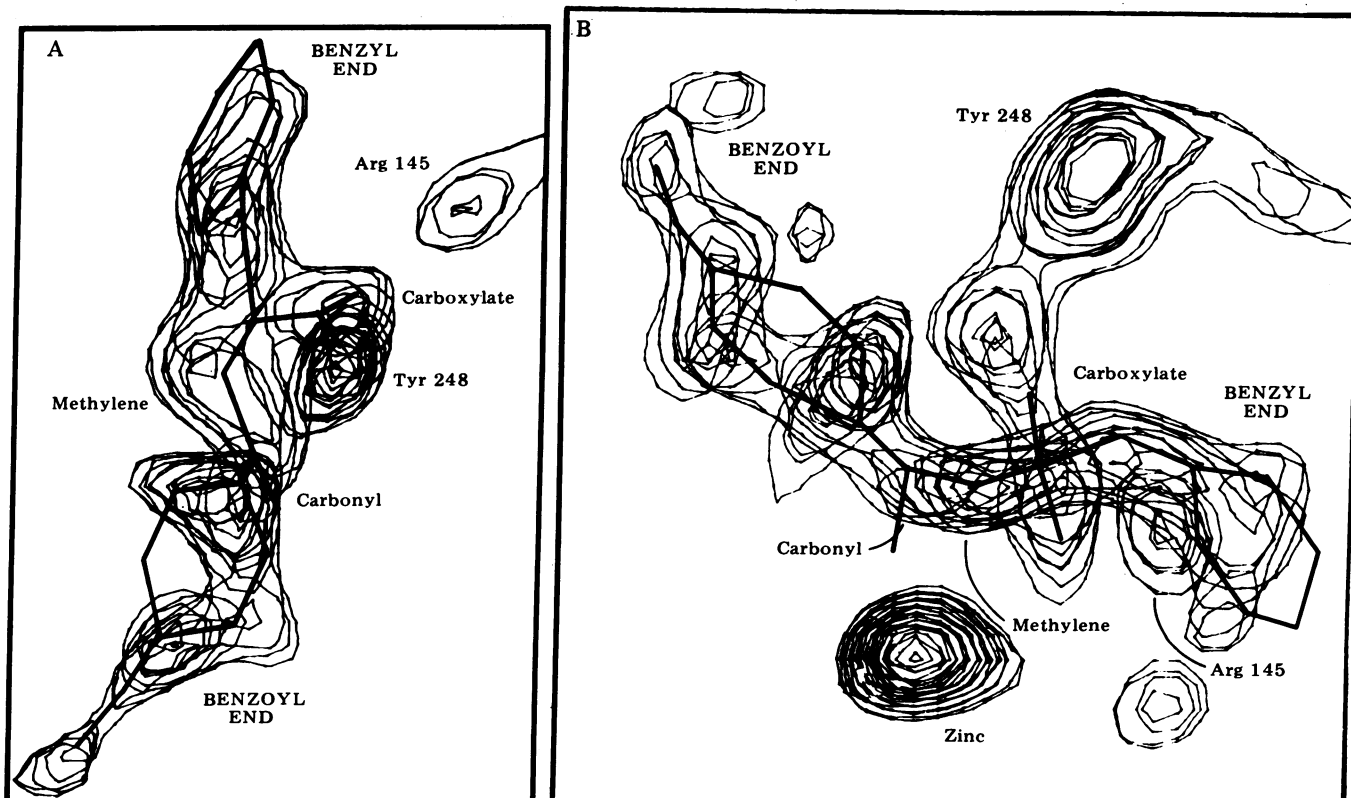

FIG. 2. Projections of the positive difference electron density map for the binding of BMBP to CPase A. (*A*) Projection down the *y* axis from *y* = 0.50 to 0.58; (*B*) projection down the *x* axis from *x* = −0.08 to 0.04. The benzoyl group appears crescent shaped due to the displacement of a water molecule in the native structure.
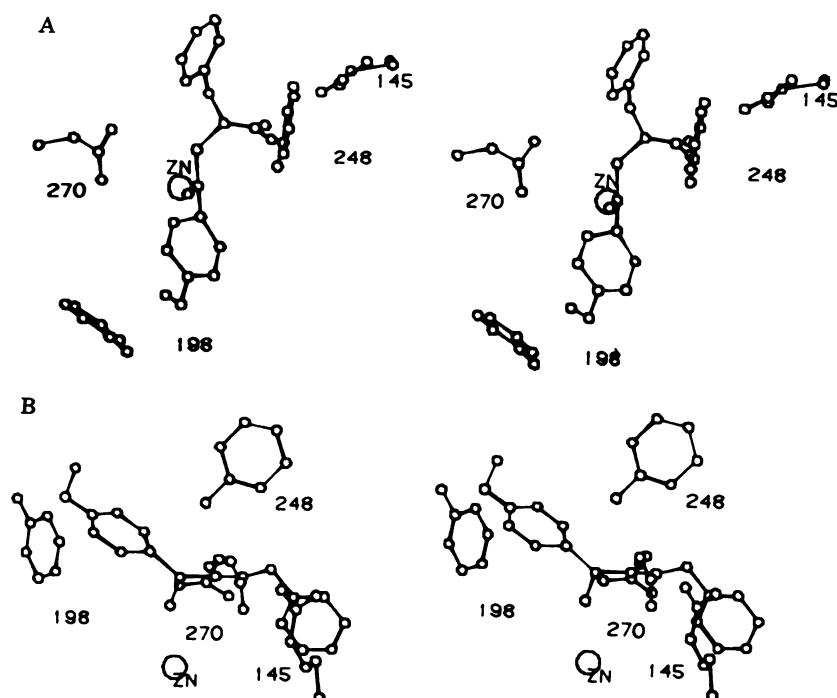
FIG. 3.   Stereoviews of the binding of BMBP to the active site of CPase A. (A) Looking down the y axis; (B) looking down the x axis. The R proton, which exchanges, is that nearest the carboxylate anion of Glu 270.

The electron density maps clearly support the mode of binding of BMBP to the active site of CPase A postulated by Sugimoto and Kaiser (5, 6). The difference map cannot exclude a role for water in proton abstraction at the methylene carbon, but no water molecules intervene between BMBP and Glu 270. Furthermore, the carbonyl oxygen of BMBP interacts directly with the zinc. Thus, an alternate mode of binding, suggested by Sugimoto and Kaiser, but not favored by them, in which the carboxylate group of Glu 270 holds the inhibitor keto group, cannot be reconciled with the electron density map. However, the crystal structure only reveals features of a statistically averaged complex and is therefore insensitive to subtle changes that may occur during the catalytic hydrogen exchange process.

BMBP bears some structural resemblance to a class of inhibitors of CPase A studied by Byers and Wolfenden (9, 10). The prototype of these inhibitors, L-benzylsuccinic acid, differs from BMBP only in the replacement of the p-methoxybenzoyl group with a carboxylate anion, which probably has enhanced binding to the positive Zn ion. The potent inhibitory effects of half esters at C_1 of L-benzylsuccinate implies a functional analogy between BMBP and this class of inhibitors. L-Benzylsuccinate also competitively inhibits the endopeptidase thermolysin, an interaction that has been characterized crystallographically by Bolognesi and Matthews (11). Since earlier work from Matthews' group revealed similarities in the active site geometries and peptide binding modes of CPase A and thermolysin (12), Bolognesi and Matthews presented a proposal for the binding of L-benzylsuccinate to CPase A which is, in general, similar to the observed binding of BMBP. However, their suggestion that Tyr 248 will interact with the second oxygen of the Zn-bound carboxylate in the L-benzylsuccinate-CPase A complex, should also include the possibility for interaction of the phenolic side chain of Tyr 248 with the inhibitor carboxylate-Arg 145 site, as observed here in the CPase A-BMBP substrate complex.

The interaction of the p-methoxy group with Tyr 198 provides a possible explanation for the effect of para substituted

benzoyl groups on nonspecific ester hydrolysis. Bunting et al. (13) have shown that although CPase A hydrolyzes para substituted O-benzoyl-2-hydroxybutanoic acids, the unsubstituted ester binds predominately in the nonproductive mode. Nonproductive interactions could involve "wrong way" binding, with the benzoyl group entering the hydrophobic pocket in place of the appropriate alcoholic side chain, or possible multiple binding. Furthermore, the benzoate ion is a competitive inhibitor of esterase activity whereas para substituted benzoate ions are noncompetitive inhibitors. Thus, para substituents may tend to direct binding to a site away from the primary hydrophobic binding region. The additional interaction of the para substituents with Tyr 198 may thus reduce the extent of wrong way nonproductive binding, thereby stabilizing the productive binding mode.

Formally, BMBP is an analogue of either a peptide or an ester substrate. However, its expected flexibility probably resembles that of an ester more closely than that of a peptide substrate in the CO-X region, where X is CH_2, O, or NH. This study therefore supports the proposal that cleavage of esters and peptides occurs in the same local region of the enzyme. The dramatic variation of binding and kinetic parameters between esters and peptides is probably therefore associated with (i) initial binding regions and (ii) with different rate-controlling stages after achievement of the binding modes found in this study and in our earlier studies of glycyltyrosine.

Finally, the present observation that BMBP binds in a fashion similar to that of glycyltyrosine and induces similar conformational rearrangements in CPase A indicates that a hydrogen bond donor or acceptor at the methylene position is unnecessary to allow these interactions in the binding mode that precedes the catalytic steps.

In summary, we can see no reasons for modifying the binding mode that precedes catalysis, as given in earlier proposals of the x-ray diffraction studies, and we believe that this study supports the binding modes proposed earlier for extended substrates. Of course, this binding mode may be the Michaelis complex, not yet fully activated toward the transition state to the first in-

termediate. At a later stage of the reaction, deacylation of the anhydride, if formed, could be promoted by attack of H_2O or OH^- which adds to Zn, as suggested earlier (2, 4). Proof of five-coordinated Zn in the deacylation step of the ester substrate, O-(p-chlorocinnamoyl)-L-β-phenyl-lactate in the −25°C to −60°C range has been reported in the Co enzyme by use of electron paramagnetic resonance methods.[†]

[†] Kuo, L. C. & Makinen, M. W., poster 27, presented at the *Conference on Methods for Determining Metal Ion Environments in Proteins*, Jan. 10–12, 1979, Las Cruces, New Mexico.

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