THE EXISTENCE OF MULTIPLE CONFORMATIONAL FORMS IN ANHYDROCHYMOTRYPSIN

Richard M. Schultz, A. Konovessi-Panayotatos

and William J. Treadway, Jr.

Department of Biochemistry and Biophysics
Loyola University Stritch School of Medicine
Maywood, Illinois 60153

Received July 15,1974

Summary

The conversion of the serine-195 in α-chymotrypsin to dehydroalanine results in two conformational substates that differ in their extinction coefficients at 240nm. The active site methionine-192 in the substate with lower absorption at 240nm is alkylated by α-bromo-4-nitroacetophenone at a rate of $7.0 \times 10^{-4} sec^{-1}$, similar to that found for α-chymotrypsin; the substate with higher absorption at 240nm reacts 14 times slower. These two substates are not separated by an affinity resin containing lima bean trypsin inhibitor. These data infer that the serine-195 plays a role in the stabilization of the active site conformation in α-chymotrypsin.

In 1966 Weiner et al.(1) reported the preparation and characterization of a form of α-Cht[1] in which the catalytically essential serine-195 is chemically converted to dehydroalanine. This catalytically inert protein, which we will designate ACht-I[1], was reported to bind small virtual substrates 3 to 8 times less strongly than α-Cht. Information obtained from the study of ACht-I was utilized in the formulation of current hypotheses of the catalytic role of the serine-195 in α-Cht (1,2).

Recently, Ako et al.(3) reported the preparation of an ACht[1] by slightly different chemical procedures and utilizing an affinity resin of agarose bound to lima bean trypsin inhibitor

---

[1]Abbreviations: α-Cht, α-chymotrypsin; ACht, anhydrochymotrypsin; ACht-I, anhydrochymotrypsin prepared by the procedure of Weiner et al.(1); ACht-II, anhydrochymotrypsin prepared by the procedure of Ako et al.(3); PMS-Cht, phenylmethylsulfonylchymotrypsin; ATEE, N-acetyl-L-tyrosine ethyl ester; BrNAP, α-bromo-4-nitroacetophenone; tris, trishydroxymethylaminomethane.

in a final purification step. ACht-II[1] was reported to differ from the previously prepared ACht-I in its greater affinity for lima bean trypsin inhibitor and its lack of a 240nm difference absorption with respect to native α-Cht. The fraction not binding to the affinity column was reported to have similar spectral properties to ACht-I.

In this communication we report further on the spectral and active site conformational properties of anhydrochymotrypsin.

Procedures

Absorption spectra were obtained on Cary-15 and Heath 707 Double Beam Recording Spectrophotometers. Circular dichroism spectra were recorded on a Durrum-Jasco ORD/CD/UV-5 Spectropolarimeter modified to a maximum sensitivity of 2×10^{-3}deg/cm.

Enzyme activity was assayed with ATEE[1] (4). Protein concentration was determined at 280nm ($\varepsilon_{280}=5.0 \times 10^4$ $\underline{M}^{-1}cm^{-1}$) (5). ACht preparations had less than 1.6% of native α-Cht activity.

Results

ACht-I was prepared according to Weiner *et al.* (1). ACht-II was prepared according to Ako *et al.* (3) by treating PMS-Cht[1] with 0.1N KOH for 1 hr at 3°C, leading to "crude" ACht. Crude ACht was chromatographed over a lima bean trypsin inhibitor affinity resin leading to 11 mg of non-binding ACht-II (eluted initially with pH 8 solution) and 29 mg of binding ACht-II (eluted subsequently with pH 2 solution) (3). Ako has shown that crude, non-binding and binding ACht-II all contain one equivalent of dehydroalanine per mole of enzyme (3). The analysis of ACht-I on the affinity resin found it to be composed of 80% non-binding and 20% binding protein.

The extinction coefficients for various preparations of ACht were determined at 240nm (Table I). We would like to herein de-

Table I. Spectral and Kinetic Data Obtained for Different Samples of Anhydrochymotrypsin

Enzyme Sample	ε_{240}[d] $10^{-4} \cdot M^{-1}cm^{-1}$	$[\theta]_{229}$[i]	% of Fast Reacting Substate[j]
ACht-I	5.0[e]	3.8	0
crude-ACht-II[a]	4.6[e]	3.7	
ACht-II(NB[b])	3.9[f]		
ACht-II(NB[b])	4.0[e]	3.6	45
ACht-II(NB[b])	4.7[g]	3.8	
ACht-II(B[c])	3.9[f]		
ACht-II(B[c])	4.9[e]		16
ACht-II(B[c])	4.0[e,h]	4.4	62
PMS-Cht	3.9[e]	4.3	

[a] ACht prior to affinity chromatography. [b] ACht not binding to affinity resin. [c] ACht retained by affinity resin at pH 8.0. [d] (E)=0.5-1.9x10^{-5} M. [e] pH 7.8, 0.05 M sodium phosphate, 0.1 M NaCl, at 25°C. [f] In mM HCl after dialysis and prior to lyophilization. [g] Different lot than sample above, pH 8.0, 0.1 M tris, 0.1 M NaCl, 0.12 M $CaCl_2$. [h] Same sample as above, refrigerated for 2 weeks. [i] Circular dichroism units of 10^3 deg-cm^2/decimole, error $\pm$ .12x10^3 deg-cm^2/decimole, solution conditions same as for ε_{240}. [j] Error $\pm$15%, see Fig. 1 for details.

---

scribe our observations on the variation in ε_{240} during the preparation of a particular lot of ACht-II. PMS-Cht (ε_{240}= 3.9x10^4 $M^{-1}cm^{-1}$) was converted to crude ACht (ε_{240}=4.6x10^4 $M^{-1}cm^{-1}$) and then separated into non-binding and binding ACht-II on the affinity column. Both products had a low 240nm absorption of 3.9x10^4 $M^{-1}cm^{-1}$ after dialysis in 1mM HCl. However, after lyophilization the absorption increased to 4.0x10^4 $M^{-1}cm^{-1}$ and 4.9x10^{-4} $M^{-1}cm^{-1}$ for non-binding and binding ACht-II, respec-

tively, in pH 7.8 phosphate buffer solution (Table I). Dialysis of the binding ACht-II against tris[1] buffer, pH 8.0, as described by Ako (3) did not change the ε_{240}. A part of the binding ACht-II with high ε_{240} was rechromatographed over the affinity column to ascertain if it had been denatured during lyophilization. All the protein bound to the column at pH 8 and came off in the binding fraction when the eluting buffer was changed to pH 2. However, after dialysis and lyophilization its ε_{240} was found to be reduced ($\varepsilon_{240}=4.05\times10^{4}$ $\underline{M}^{-1}cm^{-1}$). ACht-II, binding, with high ε_{240} was refrigerated for two weeks at 4°C, after which its ε_{240} was found to be lower (4.0×10^{4} $\underline{M}^{-1}cm^{-1}$, at pH 7.8).

Accordingly, the 240nm extinction coefficient for ACht is variable. Both the binding or non-binding protein fractions from the affinity column may exhibit a low or high ε_{240}. In our hands, it appears that the contact of the protein with the affinity resin induces a low ε_{240} and lyophilization sometimes acts to increase ε_{240}. No consistent variation of the ε_{240} absorption with enzyme concentrations between 0.13 to 1.8×10^{-5} $\underline{M}$ is observable.

The circular dichroism of α-Cht and ACht show a negative trough at 229nm. The ellipticity found for this minima for some ACht preparations are given in Table 1. The data show PMS-Cht and ACht-II (binding) have similar ellipticity. ACht-II (non-binding) and ACht-I have a slightly reduced ellipticity at 229nm than the above enzyme forms, in general agreement with the observations of Ako (3).

The active site methionine-192 in α-Cht may be specifically alkylated by BrNAP[1] leading to a product with a new absorption band at 350nm (6). We have studied the rate of methio-

nine-192 alkylation in α-Cht and ACht by BrNAP, at pH 5.6, by spectrophotometrically following the increase in absorption at 350nm. The data for ACht plotted according to the first order rate expression show the existence of a fast and slow reacting component, indicative of the presence of two enzyme conformational forms with different reactivities towards BrNAP (Fig. 1).

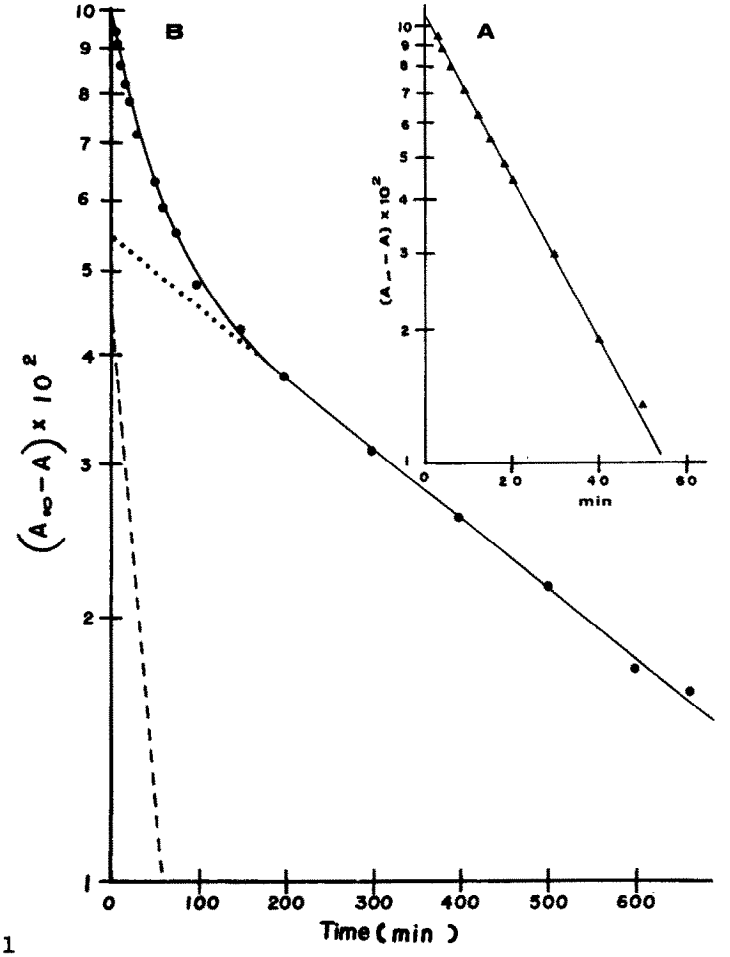

Fig. 1

Semilogarithmic Plot of Change in Absorbance at 350nm Due to Reaction of BrNAP with Native and Anhydrochymotrypsin. A. Reaction with Native α-Cht: $(E)=2.0\times10^{-5}$ M, $(BrNAP)=2.0\times10^{-4}$ M, pH 5.6, 0.05 M in sodium acetate, 25°C; $k=6.1\times10^{-4}$ sec^{-1}. B. Typical Reaction with ACht-II: $(E)=1.6\times10^{-5}$ M, $(BrNAP)=2.0\times10^{-4}$ M, same solution conditions as in A; ——— observed reaction, ···· extrapolation of slow reacting component to time zero, ---- fast reacting component calculated by substraction of slow reaction from observed reaction.

The pseudo first order rate constant found for the fast reacting component is $7.0\pm2.8\times10^{-4}sec^{-1}$, similar to that found for the reaction of BrNAP and native α-Cht. The rate constant for the reaction of BrNAP and the slower reacting component is approximately 14 times slower ($0.50\pm.20\times10^{-4}sec^{-1}$). The percent of the fast reacting species varied from 13% to 68% of the total reaction, depending on the particular sample (Table I). A greater percentage of the fast reacting species appeared to be present in samples with lower absorption at 240nm.

Discussion

Our data supports the existence of four conformational states for ACht at room temperature. One pair of conformational states is separated by their different affinities toward lima bean trypsin inhibitor. The enzyme conformation that does not bind well to lima bean inhibitor has a slightly lower circular dichroism at 229nm. These two conformational states seem not to be readily interconvertible. A second pair of conformational states are apparent from the variation in absorption at 240nm and the observation of two distinct rates for alkylation by BrNAP found in both of the conformational states differentiated by the lima bean inhibitor affinity resin. Changes in environment appear to facilitate interconversions among this second pair of substates.

The decreased rate of BrNAP modification for the slower reacting ACht conformation may be due either to a poorer binding of BrNAP by the active site or a decreased accessibility of the methionine-192 to bound BrNAP. If one assumes the decreased rate is due to a poorer binding constant, then a similar decrease in binding strength of approximately 14-fold may be predicted for the binding of other small substrate analogs to this conformational substate of ACht. The factor of increase for any one ACht sample will be dependent on the

mixture of conformational substates in that particular sample and, accordingly, should be between 1- and 14-fold. In agreement with this prediction, Weiner et al. (2) found the binding constants for substrate analogues to ACht-I to be increased between 3- and 8-fold.

These data infer that the serine-195 hydroxymethyl moiety may have a structural role, in addition to its well known catalytic role, in giving stability to the productive active site conformation of native α-Cht. Its elimination in ACht appears to result in four different conformational forms, some of which bind substrates less well than the native conformation of α-chymotrypsin.

Acknowledgement

We gratefully acknowledge the financial support of the National Institutes of Health (Grant No. Am 16733).

REFERENCES

1. Weiner, H., White, W.N., Hoare, D.G. and Koshland, D.E., Jr. (1966) J. Am. Chem. Soc. 88, 3851-3859.

2. Weiner, H., and Koshland, D.E., Jr. (1965) J. Biol. Chem. 240, PC 2764-PC 2766.

3. Ako, H., Foster, R.J., and Ryan, C.A. (1974) Biochem. 13, 132-139.

4. Schwert, G.W. and Takenaka, Y. (1955) Biochem. Biophys. Acta 16, 570-575.

5. Dixon, G.H., and Neurath, H. (1957) J. Biol. Chem. 225, 1049-1059.

6. Sigman, D.S., Torchia, D.A., and Blout, E.R. (1969) Biochem. 8, 4560-4566.