STUDIES ON THE INFLUENCE OF CARBOXYL-TERMINAL AMINO ACID RESIDUES ON THE ACTIVITY AND STABILITY OF HUMAN ERYTHROCYTE CARBONIC ANHYDRASE B

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1. Introduction

Carbonic anhydrase B (HCAB)* is the dominating form of carbonic anhydrase in human red cells [1]. This form and the other main isoenzyme, called C, from human erythrocytes have been structurally well characterized. The complete amino acid sequences are known for both proteins [2,3] and their three-dimensional structures have been determined with X-ray diffraction to high resolution ([4–6] K. K. Kannan et al., to be published).

The access of structural information makes the human carbonic anhydrase an attractive system for studies of structure-function relationships. One technique used in similar studies on other enzymes has been to modify the protein structure by proteolytic degradations. Carboxypeptidase has been used on several enzymes to remove one or a few amino acid residues from the carboxyl-terminal end [7–10].

In this paper we describe an investigation on the influence of the carboxyl-terminal region on the properties of HCAB. The carboxyl-terminal region in this protein has the following sequence: Arg_{254}–Thr–Val_{256}–Arg–Ala_{258}–Ser–Phe_{260}OH. In its native state the enzyme is only poorly attacked by carboxypeptidase [11]. Since conditions for reactivating the denatured carbonic anhydrase has recently been developed [12] it was tempting to investigate the possibilities to digest the enzyme in the denatured state and subsequently try to reactivate the product.

* *Abbreviations:* HCAB = human erythrocyte carbonic anhydrase B; GuHCl = guanidine hydrochloride; DFP = di-isopropyl phosphorofluoridate.

In this way we obtained two rather well-defined derivatives of HCAB. The carboxylterminal phenylalanine residue, Phe_{260}, could be specifically removed with carboxypeptidase A to yield Des(260)HCAB. A more extensive digestion releases Phe_{260}, Ser_{259}, and Ala_{258} giving Des(258–260)HCAB. Des(260)-HCAB and Des(258–260)HCAB could be successfully renatured and was found to possess at least 90% of enzymic activity. Des(258–260)HCAB is considerably less stable in guanidine hydrochloride (GuHCl)* than the native HCAB. These findings would suggest that the carboxyl-terminal region of the enzyme is not of critical importance for enzymic activity but would contribute to the stability of the catalytically active conformation.

2. Materials and methods

HCAB was prepared according to Henderson and Henriksson [13]. GuHCl of sequential grade was obtained from Pierce Chemical Co. Carboxypeptidase A was a DFP*-treated preparation (COADFP) purchased from Worthington Biochemical Co., iodoacetamide (sodium salt) from Sigma Chemical Co. was recrystallized from water.

Amino acid analyses were carried out according to Moore and Stein [14] using a Beckman 120 B amino acid analyzer. Esterase activity was determined spectrophotometrically by the method of Whitney et al. [15], using *p*-nitrophenyl acetate as substrate. Carbon dioxide hydration activity was measured by the colorimetric procedure described by Rickli et al. [16]. The concentration of HCAB was determined by absorbance measurements at 280 nm using a value of

$A_{280}^{0.1\%}$ of 1.63 [17]. Measurements of circular dichroism were carried out using a JASCO J-20 spectropolarimeter.

Des(260)HCAB was prepared as follows. Lyophilized HCAB, 45 mg, was dissolved in 1 ml 5 M GuHCl–0.1 M $NaHCO_3$. The solution was diluted with 0.1 M $NaHCO_3$ to 1 M GuHCl. Under these conditions the rate of refolding of the enzyme is slow [12] and carboxypeptidase A (enzyme:substrate ratio 1:200) was immediately added. The digestion was allowed to proceed at 37°C for 5 min. For the determination of amino acids released an aliquot was taken out and diluted with 0.2 M sodium citrate buffer, pH 2.2, for amino acid analysis.

In order to renature the digested HCAB the concentration of GuHCl has to be raised. This is believed to push the equilibrium for the 'incorrectly' folded states (see ref. [18]) which are kinetically inert to reactivation towards the random-coil state [12]. The solution of digested HCAB was made 5 M with respect to GuHCl by adding 0.1 M Tris–sulfate, pH 7.5, containing GuHCl. In this way the enzyme was diluted not more than 10 times. Mercaptoethanol (0.1 mg) was added and the enzyme, which contains a single sulfhydryl group, was carboxamidomethylated by allowing it to react with 5 mg iodoacetamide. After 2 hr at room temperature in the dark the reaction was stopped by adding 20 mg mercaptoethanol.

Reactivation of the enzyme was accomplished as described by Carlsson et al. [12] by diluting the solution to 0.5 M GuHCl–0.1 M Tris–sulfate, pH 7.5, and 0.025 mg protein/ml. The reactivation was followed by measurements of carbon dioxide hydration activity. After dialysis against 0.02 M $NaHCO_3$ the enzyme was concentrated by pressure dialysis. Active Des(260)HCAB was isolated from the reaction mixture using affinity chromatography carried out in 0.1 M Tris–sulfate, pH 7.5 and with buffer containing 0.1 M NaI as the elutant [19]. The recovery of enzyme was approximately 20%.

The Des(258–260)HCAB was prepared in a similar manner but the carboxypeptidase:substrate ratio was 1:5 and the time of digestion was 1 hr. The recovery of enzyme after affinity chromatography and dialysis was approximately 25%.

The stability of the derivatives of HCAB obtained as described above was investigated by measuring the carbon dioxide hydration activity after incubation for 24 hr at various concentrations of GuHCl. The experiments were carried out in 0.1 M Tris–sulfate, pH 7.5 at room temperature, and the concentration of enzyme was 0.05–0.10 mg/ml [12].

3. Results

The results of the digestions of HCAB with carboxypeptidase A are shown in table 1. As can be seen

Table 1
Release of carboxyl-terminal amino acid residues from human carbonic anhydrase B (HCAB) on digestion with carboxypeptidase A and the specific enzymic activities of the products obtained

Main product	Amino acids released (moles/mole of HCAB)				Specific enzymic activity (relative to undigested HCAB)	
	Phe	Ser	Ala	Arg, Val, Thr	Carbon dioxide hydration	Esterase reaction
Des(260)HCAB	0.93	< 0.05	< 0.05	–	1.0	1.0
Des(258–260)HCAB	1.00	1.00	0.93	< 0.05	0.89	0.90

The release of amino acids were calculated from amino acid analyses and protein concentrations estimated by spectrophotometric measurements (A_{280}). The carboxypeptidase digestions were carried out under denaturing conditions and the reaction products were subsequently carboxamidomethylated, renatured, and purified by affinity chromatography. Their specific enzymic activities were measured after removal of inhibitory NaI by dialysis. The values of the derivatives were compared with the specific activity of carboxamidomethylated, undigested HCAB which is essentially the same as for HCAB having the sulfhydryl group unblocked (Carlsson et al., to be published). For further experimental details, see the section Materials and methods.

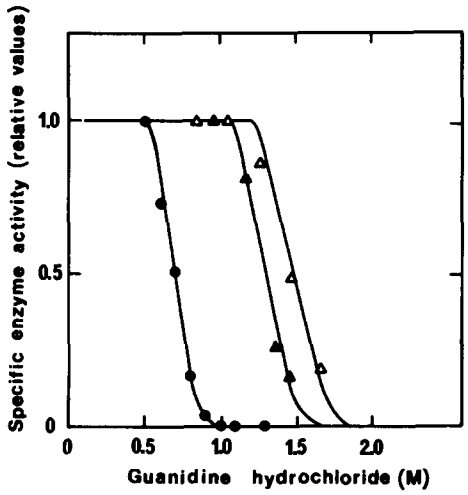

Fig. 1. Stabilities of human carbonic anhydrase B (HCAB) and two derivatives of the enzyme obtained by digestion with carboxypeptidase A. The relative specific enzymic activities in the carbon dioxide hydration reaction were measured after incubation for 24 hr at different concentrations of GuHCl in 0.1 M Tris–sulfate, pH 7.5. The enzyme derivatives and the undigested enzyme were all carboxamidomethylated. The undigested, carboxamidomethylated HCAB has a stability similar to that of native HCAB (Carlsson et al., to be published). For experimental details, see section Materials and methods. (△) HCAB (undigested); (●) Des(258–260)HCAB; (▲) Des(260)HCAB.

the milder conditions of digestion, used in the preparation of Des(260)HCAB, release the terminal Phe_{260} in a yield of 93% while the residues coming next in the sequence, Ser_{259} and Ala_{258}, are liberated to less than 5%. It is interesting to note that a different procedure for denaturation of HCAB used in an earlier digestion [11] did not allow any discrimination in the rate of liberation between Phe_{260}, Ser_{259}, and Ala_{258}.

Under the more severe conditions of digestion used here for preparing Des(258–260)HCAB the two first residues are liberated to 100% while the third residue, Ala_{258}, is released to 93%. Arg_{257}, coming next to the alanine residue, is very slowly attacked in accordance with previous experience [11].

The relative specific enzyme activities measured after affinity chromatography and dialysis are also shown in table 1. The values obtained are comparable with that of the native HCAB suggesting that Des(260)-HCAB and Des(258–260)HCAB when renatured have attained a conformation closely similar to that of native HCAB. This is further supported by the circular dichroism spectrum of Des(260)HCAB which was found to be within experimental errors identical with that of native HCAB.

The stabilities of the enzyme derivatives in GuHCl are illustrated in fig. 1. It is evident that Des(258–260)-HCAB has a markedly decreased stability compared with the undigested HCAB.

4. Discussion

It can be concluded from this study that the reactivation of denatured HCAB to essentially fully active enzyme is possible when 1 to 3 amino acid residues have been removed from the carboxyl-terminal end of the enzyme. These residues are obviously not critical for enzymic activity. It was once proposed [20] that the carboxyl-terminal end is located in the active site pointing towards the zinc ion (see also ref. [21]). This view became questioned, however, on the basis of amino acid sequence data showing that the 5–6 first carboxyl-terminal residues in different forms of carbonic anhydrase are rather variable [11]. In 1972 Liljas et al. [4] published a three-dimensional model for human carbonic anhydrase C from 2.0 Å resolution X-ray diffraction data. The interpretation of this model has been partly revised [5] using the knowledge of the amino acid sequence which later on became available [3]. Recently, high resolution X-ray data have also been achieved for HCAB ([6]; K. K. Kannan et al., to be published). The structural studies show that the conformations of the B and C enzymes are very similar while the amino acid sequences differ to about 40% [3]. In both enzymes, the carboxyl-terminal region is located rather much on the surface of the molecule quite distant from the active site region. This situation appears consistent with the finding in the present investigation that the residues in this region are not critical for the catalytic function of the enzyme.

The stabilities of the derivatives of HCAB obtained in the present study were tested at various concentrations of GuHCl. The results are illustrated in fig. 1. The removal of the terminal Phe_{260} seems to cause a small shift in stability towards lower concentrations

of GuHCl as compared with the undigested HCAB. When Ser_{259} and Ala_{258} are also removed, giving Des(258–260)HCAB, the stability is further decreased. These findings point towards a structural role for the carboxyl-terminal region. The lowered stability observed in GuHCl (fig. 1) may reflect a lowered stability even under physiological conditions. Thus, one function of the carboxyl-terminal residues as suggested from the present investigation would be to contribute to the stability of the biologically active conformation of the enzyme molecule. This would mean a selective advantage, justifying the presence of these residues in the protein.

The structural role for the individual carboxyl-terminal residues in HCAB, suggested by the data in fig. 1, can be discussed in detail using the three-dimensional model of carbonic anhydrase. From the X-ray work [4–6] it has been proposed that the carboxyl-terminal region in both enzymes is involved in the secondary structure of the protein. A striking feature in the conformation of carbonic anhydrase is an extensive pleated-sheet arrangement forming a core through the slightly elongated enzyme molecule. The pleated-sheet is built up of ten chains with the carboxyl-terminal region forming a penultimate chain in this arrangement. In both forms of carbonic anhydrase the pleated-sheet has been interpreted to comprise four amino acid residues of the carboxyl-terminal sequence. In HCAB these residues are Val_{256}, Arg_{257}, Ala_{258}, and Ser_{259} [6]. The terminal residue, Phe_{260}, does not partake in the pleated-sheet structure and when this residue is specifically removed there is a rather inconsiderable drop in stability as illustrated in fig. 1. From the X-ray model, Phe_{260} has been quoted to partake in a cluster of aromatic amino acid residues [6] but the results obtained in the present study suggest that this interaction does not contribute significantly to stabilize the native conformation. When Ser_{259} and Ala_{258} are removed together with Phe_{260} a larger decrease in stability occurs (see fig. 1). This finding would be consistent with a specific role for Ser_{259} and Ala_{258} in the secondary structure of the protein of the kind predicted from the X-ray work.

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