THE C-TERMINAL FRAGMENT OF HUMAN GLUTATHIONE REDUCTASE CONTAINS THE POSTULATED CATALYTIC HISTIDINE

Renate UNTUCHT-GRAU, Georg E. SCHULZ and R. Heiner SCHIRMER
Max-Planck-Institut fur medizinische Forschung, Jahnstr. 29, 6900 Heidelberg, FRG

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1. Introduction

The dimeric flavoenzyme glutathione reductase (mol. wt 100 000) [1,2] catalyses the reaction:

$$NADPH + GSSG + H^+ \rightleftharpoons NADP^+ + 2\ GSH$$

The amino acid sequence around the catalytic disulfide in human glutathione reductase is similar to that of other disulfide reductases [1,3]. Spectroscopic data indicate that intermediate states during catalysis are common to lipoamide dehydrogenase (EC 1.6.4.3) and glutathione reductase (EC 1.6.4.2), notably a charge transfer complex between a thiolate ion and the (re)oxidized flavin [4–6]. For stabilizing this thiolate, a protonated base was proposed [7,8], in analogy to the ion pair Cys^-/His^+ in papain [9,10]. By X-ray diffraction analysis of human glutathione reductase, the redox-active disulfide peptide was found to be in contact with the flavin [11,12]. Near Cys-46, the proposed thiolate ion, the sidechain of residue 450 of the other subunit was found in the electron density map. From size and shape it was interpreted as a His [12]. The intention of this study was to sequence the corresponding peptide, to locate it in the electron density map, and to chemically identify residue 450.

2. Materials and methods

2.1. *Isolation, properties and sequence analysis of the fragment*

CNBr-produced fragments of carboxymethylated glutathione reductase were citraconylated and separated by gel filtration. The fragments of lower molecular weight (represented in fractions T, U, V and W, of [3]) were desalted, decitraconylated and subjected to peptide map analysis [13,14]. Amino acid analyses of the separated fragments revealed that only one fragment, the so-called H-peptide containing 25 amino acids, did not possess homoserine. Consequently, this fragment was assumed to represent the C-terminal section of glutathione reductase. On a preparative scale the H-peptide was purified from 2 μmol desalted and decitraconylated fraction U [3] by high voltage paper electrophoresis at pH 6.5 (μ_{Asp} of the H-peptide = 0.2) and by descending paper chromatography in butan-1-ol/acetic acid/water/pyridine (15/3/10/12, by vol.). The R_F value of the H-peptide in this system was 0.4. The sequence determination is described in fig.1 (see also [15]).

2.2. *Fitting of the sequenced H-peptide to the electron density map of glutathione reductase*

As described in [12], the course and the direction of the total polypeptide chain of 462 residues was traced in the electron density map by visual inspection. Then all amino acid residues were read from the electron density map yielding the so-called 'map-sequence'. Similarity indices (table 1) were introduced which allow for the most common reading errors.

For locating the H-peptide in the map-sequence we used a computer program. As a first step this program aligns the H-peptide with residues 1–25 of the map-sequence. For each pair of the aligned 25 residues the similarity index is taken from table 1. The sum of the 25 similarity indices is the first A score for the comparison between H-peptide and map-sequence. In the

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Table 1
Similarity indices of amino-acid residues in electron density maps of 3 Å resolution

Ala = A	10																			
Cys = C	0	20																		
Asp = D	0	5	10																	
Glu = E	5	0	5	10																
Phe = F	0	5	0	0	20															
Gly = G	10	0	0	0	0	10														
His = H	0	5	5	0	15	0	20													
Ile = I	0	5	0	0	5	0	5	15												
Lys = K	5	0	5	5	0	5	0	0	10											
Leu = L	0	0	0	0	10	0	5	10	0	10										
Met = M	0	5	0	0	10	0	10	5	0	5	20									
Asn = N	0	5	5	5	0	5	5	0	5	5	0	10								
Pro = P	5	0	0	0	5	5	0	0	0	0	0	5	10							
Gln = Q	0	0	5	5	5	0	5	5	5	5	5	5	0	10						
Arg = R	5	0	5	5	0	5	5	0	10	0	0	5	0	5	10					
Ser = S	5	5	5	5	0	5	0	0	5	0	0	5	5	0	5	10				
Thr = T	5	5	5	0	0	0	0	5	0	0	5	5	5	5	5	10	10			
Val = V	5	0	0	0	5	0	0	10	0	10	0	10	5	0	0	5	10	15		
Trp = W	0	5	0	0	15	0	10	5	0	0	5	0	0	0	0	0	0	0	30	
Tyr = Y	0	5	0	0	15	0	15	5	0	0	10	0	0	5	5	0	0	0	15	20
	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y

The matrix reflects the most common errors when interpreting electron densities in terms of an amino acid sequence. Let us take the last line as an example: If the density representing a Tyr is correctly read as a Tyr, the similarity index is as high as 20; if it is read as a Met the value is only 10 and if it is read as a Ser the value is 0. Figure 1B shows that 8 out of 25 residues were read correctly from the electron density map (which is in the range of sequencing success reported for similar maps of other proteins [16])

following steps *A* scores are derived for all 462–25 + 1 possible alignments between H-peptide and map-sequence. The frequency distribution of *A* scores is then analysed (fig.2).

Frame shifts caused by erroneously missing or additional residues in the map-sequence affect the *A* scores appreciably. They even may conceal the correct peptide location or the correct position of a given residue in the peptide. Therefore the computer carried out two further series of comparisons. In the *I* series we assume that there is a single erroneous insertion at any of the 25–1 positions of the overlaid section of the map sequence. The corresponding scores are computed, the highest one being the *I* score. In an analogous procedure, the *D* series, we take into account that the map-sequence may contain a single erroneous deletion; in this case the highest score is the *D* score. The frequency distribution of the resulting *I* and *D* scores is then analysed (fig.2). All these distributions are nearly Gaussian so that the computation of a standard deviation is meaningful. In each distribution the highest score represents the best location of the H-peptide. The corresponding 'quality of fit' can be expressed as the difference between the score and the mean of the distribution and is given in standard deviation units (fig.2). The degree of ambiguity of an assignment can be judged by comparing the quality of fit for the best and for the second best location.

3. Results and discussion

3.1. *The H-peptide as the C-terminal fragment of glutathione reductase*

The fact that the CNBr-produced H-peptide contains no C-terminal Hse, and the comparison of the amino acids released by carboxypeptidases from intact glutathione reductase and from the H-peptide (fig.1A) show that the H-peptide is localized at the C-terminus of the polypeptide chain. The sequence of the H-peptide as determined by standard techni-

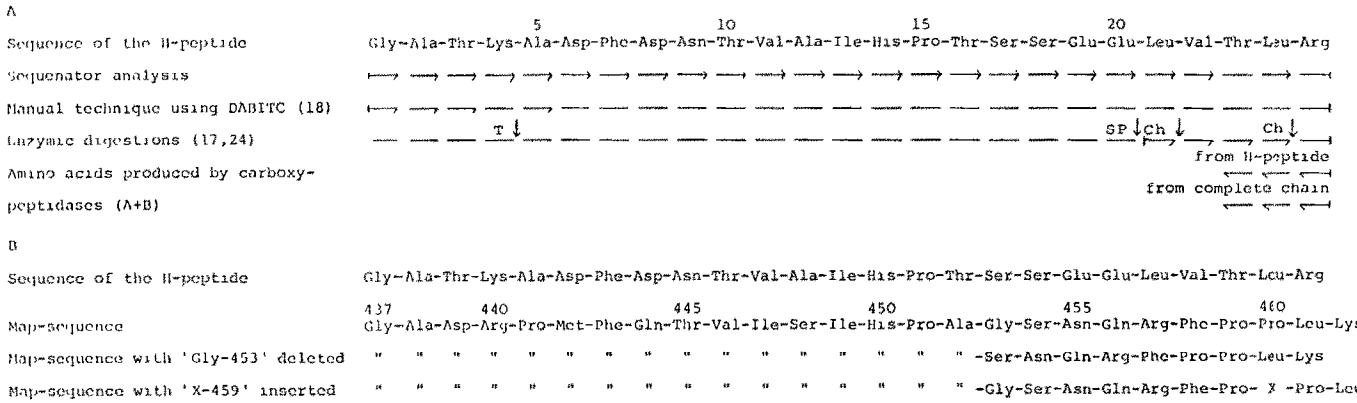

Fig.1. (A) Sequence analysis of the H-peptide. Enzymic digestions of the H-peptide and of intact glutathione reductase were carried out as in [3,17]. The amino acid sequence of the H-peptide (and one subfragment) was determined by the manual method [18] and by automated Edman-degradation in a solid-phase sequenator [19] using carbodiimide for coupling the peptide to amino-polystyrene resin or to aminopropyl glass [20–22]. The phenylthiohydantoins produced by the sequenator were determined by high-pressure liquid chromatography [23]. (——) The residue was present in the amino acid analysis of the peptide delineated by vertical lines; (horizontal arrows) the residue was positioned by the indicated methods; (vertical arrows) the cleavage sites of trypsin (T), Drapeaú [24] Staphylococcal protease (SP) and chymotrypsin (Ch). The products of each enzymic cleavage were separated and analysed according to [13,14]. (B) Alignment of the H-peptide (line 1) and the C-terminal part of the map-sequence (line 2). The bottom lines represent the best map-sequences with a single deletion and a single insertion corresponding to the highest *D* score and *I* score, respectively. The quality of fit in standard deviation units is given for each of these alignments. The values in parentheses neglect the contribution of His 450 to the quality of fit. A position 450 we had anticipated a His for chemical reasons so that the interpretation of the electron density map might have been biased at this position.

ques is given in fig.1A. At this point we could have been content with fitting this sequence to the C-terminal chain segment in the electron density map merely by visual comparison. However, at 3 Å resolution of an electron density map side chain shapes are not well enough defined [25] so that a comparison between the map and a chemically determined sequence tends to be subjective. Therefore, we used an unbiased computer program as described above.

As shown in fig.2 the 3 resulting frequency distributions of scores are approximately Gaussian. Each of the 3 distributions contains one score which deviates significantly from the mean to the positive. In each case the peptide corresponding to this high score starts at position 437 of the map-sequence

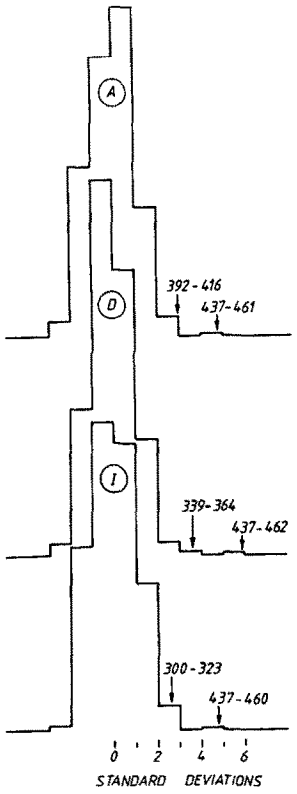

Fig.2. Frequency distribution of *A* scores, *D* scores and *I* scores. Each distribution contains 462−25 + 1 scores representing all possible locations of the H-peptide (of 25 residues) in the map-sequence (of 462 residues). In all 3 distributions the best overlay starts at position 437 (the second best at positions 392, 339 and 300, respectively).

(fig.1B). The quality of fit for the second best score (representing the fit to another chain segment) is always about 2 standard deviations lower (fig.2); consequently it is clear that the H-peptide starts at position 437. A more detailed analysis shows that the alignment which omits 'Gly-453' in the map-sequence has the highest quality of fit (5.9 standard deviations above the mean (fig.1B)). Moreover, only this alignment corresponds to the correct number of residues between position 437 and the C-terminal residue in the map. A reinspection of the electron density map revealed that the chain course could be equally well traced without 'Gly-453'. Consequently, the identification of residue 450 in the map-sequence as a His appears to be safe. It remains to be clarified whether residue 450 is identical with the essential His found by labelling glutathione reductase with ethoxyformic anhydride [26].

4. Conclusions

A computer program was used for locating the C-terminal CNBr-produced fragment in the electron density map of glutathione reductase. This program seems to be suitable for all those proteins for which a crystal structure but only fragmentary sequence information is available [27]. In the case of glutathione reductase we could establish that the conspicuous residue 450, which contacts the flavin ring via its backbone moiety and the redox-active cysteine residues at the catalytic site via its side chain, is a His. This finding supports the proposed catalytic mechanism [6,11,26] which assumes a thiol-plus-substrate activation [28] by a histidine residue.

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