Human liver glutathione *S*-transferase ψ

Chemical characterization and secondary-structure comparison with other mammalian glutathione *S*-transferases

Shivendra V. SINGH, Alexander KUROSOKY and Yogesh C. AWASTHI*

Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX 77550, U.S.A.

The isolation and chemical characterization of the anionic human liver glutathione *S*-transferase (GST) ψ (pI 5.5) are described and compared with other GST isoenzymes reported for rat and human. Amino acid compositional analysis, substrate specificity and isoelectric focusing indicated that GST ψ is a unique isoenzyme form of GST. Strikingly, however, amino acid sequence analysis of the *N*-terminal region indicated that GST ψ was identical with GST μ in the first 23 amino acid residues reported. It is likely that these two enzyme forms are at least partially structurally related. In order to investigate further the genetic relationship of GST ψ to other reported GST isoenzymes, secondary-structure analysis was performed. Despite substantial differences in the *N*-terminal-region amino acid sequences of some of the GST isoenzymes, the secondary structure of all the isoenzymes is highly conserved at their *N*-termini. The general uniformity of the secondary structure of this enzyme class at their *N*-termini strongly indicated that the observed diversity of these isoenzymes probably occurred as a result of a mechanism of gene duplication followed by divergence rather than a mechanism of convergent evolution.

INTRODUCTION

Glutathione *S*-transferases (GSTs; EC 2.5.1.18) are a family of multifunctional enzymes that play a significant role in the detoxification of a number of xenobiotics through several different mechanisms (Booth *et al.*, 1961; Jakoby, 1978; Chasseaud, 1979). GST exists in multiple forms in most mammalian tissues (for reviews, see Mannervik, 1985; Awasthi & Singh, 1985) examined so far. A number of GST isoenzymes that vary in pI values and substrate specificities have been characterized in human liver (Kamisaka *et al.*, 1975; Awasthi *et al.*, 1980; Warholm *et al.*, 1983; Hayes *et al.*, 1983; Stockman *et al.*, 1985; Singh *et al.*, 1985; Vander Jagt *et al.*, 1985). Genetic models (Board, 1981; Laisney *et al.*, 1984; Strange *et al.*, 1984, 1985) suggest that human GST isoenzymes are products of at least three autosomal loci, namely GST1, GST2 and GST3. The GST1 locus, attributed to the near-neutral isoenzymes (Strange *et al.*, 1984, 1985) such as GST μ , is polymorphic. Genetic polymorphism at the GST2 locus, attributed to the cationic isoenzymes suggested by Board (1981), has not been confirmed in subsequent studies by Laisney *et al.* (1984) and Strange *et al.* (1985). The GST3 locus, attributed to the anionic isoenzymes, is also not polymorphic. It is important that these genetic models be tested through structural and immunological studies with the purified GST isoenzymes.

Structural studies from our laboratory (Singh *et al.*, 1985) suggested that at least four immunologically and functionally distinct subunits are present among the various GST isoenzymes of human liver, indicating a minimum of four genes. Those studies (Singh *et al.*, 1985) have shown that, in the five cationic GST isoenzymes of

human liver (α , β , γ , δ and ϵ) described first by Kamisaka *et al.* (1975), two types of immunologically distinct subunits, designated as A-type (M_r 26 500) and B-type (M_r 24 500), are present. On the basis of two-dimensional polyacrylamide-gel-electrophoretic analysis, Singh *et al.* (1985) have further demonstrated that both A- and B-type subunits have two charge isomers each. It has been suggested that the cationic GST isoenzymes of human liver arise from different hetero- and homo-dimeric combinations of these subunits. In another study, Stockman *et al.* (1985) have demonstrated that two distinct M_r -26 000 subunits (B_1 and B_2) are present in the cationic GST isoenzymes of human liver and have shown that GST δ has a subunit composition of B_1B_2 . These studies also point out heterogeneity in the subunits of cationic GST isoenzymes. Further studies are needed to characterize these subunits, and a need for a unified revised nomenclature for human GST isoenzymes must be advocated at this stage. The anionic enzyme GST ω (pI 4.5; Awasthi *et al.*, 1980) is a heterodimer of M_r -24 500 (B-type) and M_r -22 500 (C-type) subunits (Dao *et al.*, 1982). However, Pattinson (1981) has reported an anionic isoenzyme from human liver which is a dimer of M_r -23 000 subunits. Another anionic enzyme, GST ψ (pI 5.5), has also been described in human liver (Awasthi *et al.*, 1980). This isoenzyme is a dimer of M_r -26 500 subunits, but these subunits are immunologically and functionally distinct from the M_r -26 500 subunits (A-type) present in the cationic isoenzymes (Singh *et al.*, 1985). The subunits present in GST ψ have therefore been designated as A' (Singh *et al.*, 1985). A near-neutral GST isoenzyme, GST μ , has also been described by Warholm *et al.* (1983) in human liver.

Abbreviation used: GST, glutathione *S*-transferase.

* To whom correspondence and reprint requests should be addressed.

This enzyme is a dimer of M_r -26300 subunits and is immunologically distinct from the cationic enzymes.

In order to understand the genetic and structural relationships among various GST isoenzymes of human liver and other tissues, the covalent structures of the different subunits of GST isoenzymes must be determined. As yet, little primary-structure information is available on the human GST isoenzymes/subunits (Dao *et al.*, 1984; Alin *et al.*, 1985). In the present paper we report the *N*-terminal-region amino acid sequence of the A' subunits (M_r 26500) of GST ψ and compare it with known amino acid sequences of other GST isoenzymes isolated from human and rat tissues. In view of the diversity of the genes coding for the GST isoenzymes, it is reasonable to question whether or not the diversity arose from gene duplication and divergence, or whether a convergent mechanism of evolution applies. This question is especially relevant when considering those GST isoenzymes that exhibit low amounts of sequence identity. Additional evidence to define the evolutionary relatedness of the GST isoenzymes can be obtained by comparing the secondary structures and, of course, ultimately the tertiary structures of these proteins. In this paper we have examined the secondary structure relatedness of GST ψ to various other reported GST amino acid sequences, to define further the evolutionary relationship of these isoenzymes. The results of these studies provide strong evidence for highly conserved secondary structure in the *N*-terminal region of human and rat GST isoenzymes reported to date.

MATERIALS AND METHODS

Materials

Human liver samples were collected at autopsy from subjects with no diagnosed disorders of the liver. The samples were cleaned of connective tissues and stored at -20°C until used. Reagents for the sequence analysis were obtained from Applied Bio-Systems. Ultra-Sphere-ODS C_{18} reversed-phase columns (25 cm $\times$ 4.6 cm) for phenylthiohydantoin analysis were obtained from Beckman Instruments. Norganic (charcoal) water-filtration cartridges were purchased from Millipore Corp. All other chemicals used were the same as described previously (Singh *et al.*, 1985).

Purification of GST ψ (pI 5.5) from human liver

GST ψ was purified by the procedure described by us previously (Singh *et al.*, 1985). Altogether six different human liver samples were analysed, and GST ψ was obtained as an apparently homogeneous preparation. The amino acid sequence of GST ψ was obtained from the enzyme purified from two different liver samples. In a typical protocol of purification, a 10% (w/v) homogenate of liver was prepared in 10 mM-potassium phosphate buffer, pH 7.0, containing 1.4 mM-2-mercaptoethanol (buffer A). The homogenate was centrifuged at 27000 *g* for 45 min and subjected to affinity chromatography (Simons & Vander Jagt, 1977). A column (1 cm $\times$ 10 cm) of GSH linked to epoxy-activated Sepharose 6B was equilibrated with 22 mM-potassium phosphate buffer, pH 7.0, containing 1.4 mM-2-mercaptoethanol (buffer B) at a flow rate of 10 ml/h, and this flow rate was maintained throughout the affinity

chromatography. After sample application, the column was washed thoroughly with buffer B, and the enzyme was eluted with 5 mM-GSH in 50 mM-Tris/HCl buffer, pH 9.6, containing 1.4 mM-2-mercaptoethanol. The eluted enzyme was dialysed against buffer A and subjected to isoelectric focusing in an LKB 8100-1 column with Ampholines in the pH range 3.5–10 in a 0–50% (w/v) sucrose density gradient. GST ψ appeared as a distinct peak at pH 5.5, which was pooled and re-electrofocussed. A single peak of enzyme activity was obtained corresponding to pI 5.5. The enzyme preparation used in structural studies was dialysed against double-distilled water containing 0.1% acetic acid. Apparent homogeneity of the enzyme was established when a single coincident symmetrical peak of GST activity and protein was observed during gel filtration through a Sephadex G-100 column and a single protein band was observed on electrophoresis in an overloaded urea/SDS/polyacrylamide gel in reducing conditions.

Electrophoresis

Urea/SDS/2-mercaptoethanol/polyacrylamide-slab-gel electrophoresis was performed by the method of Laemmli (1970). Two-dimensional polyacrylamide-gel electrophoresis was performed by the method of O'Farrell (1975).

Amino acid analysis

Samples were hydrolysed with 5.7 M-HCl *in vacuo* at 107°C for 24 h, 48 h and 96 h. Amino acid compositional analysis was carried out with a Beckman 121 M analyser, by using single-column methodology on Beckman AA20 resin (Duffy *et al.*, 1981) and on a Beckman 6300 analyser.

Automated amino acid sequence analysis

The primary structure of the protein was determined by micro-sequence analysis with an Applied Bio-Systems model 470 A protein/peptide sequencer. The analysis was repeated once and the repetitive yield was about 95% during both the runs. The phenylthiohydantoin derivatives of the amino acids obtained from the sequencer were identified and quantified by h.p.l.c., by C_{18} reverse-phase chromatography similar to that described by Zimmerman *et al.* (1977), with the slight modifications described below. The column temperature was maintained at 30°C . The derivatives were eluted with a gradient of solvent A (279 μl of acetic acid added to 950 ml of distilled water, pH adjusted to 5.0 with NaOH, and then 50 ml of tetrahydrofuran added) and solvent B (acetonitrile) at a flow rate of 1.3 ml/min, and the gradient elution was completed in 45 min. The column was pumped with 100% solvent A for 2 min after injection and then programmed to reach 44% of solvent B at 26 min. The concentration of solvent B was then returned to 0% at 38 min. Finally, the column was equilibrated with 100% of solvent A up to 45 min. Stable h.p.l.c. baselines at higher sensitivities were obtained by using filtered water as described by Nagle *et al.* (1986).

Secondary-structure analysis

The secondary structure of the protein was predicted by the method of Chou & Fasman (1978).

Fig. 1. Isoelectric-focusing profiles of 27000 *g* supernatants of 10% (w/v) homogenates of human liver

The isoelectric focusing was performed with Ampholines in the pH range 3.5–10 by the procedure given in the Materials and methods section. ○, pH gradient; ●, GST activity with chlorodinitrobenzene as the electrophilic substrate. The numbers above peaks indicate isoelectric points of the isoenzymes.

RESULTS

Isolation of GST ψ

GST ψ was purified from six different human liver samples. Fig. 1 presents the isoelectric-focusing profiles of the 27000 *g* supernatants of the homogenates of six different human liver samples. The isoelectric-focusing profiles of the supernatants were similar to the corresponding isoelectric-focusing profiles of affinity-purified GST from these six samples (results not shown). As shown in Fig. 1, GST ψ was present in significant amounts in the samples III, IV and V and was practically absent from the samples I, II and VI. GST ψ represented less than 10% of total GST activity of human liver, with the exception of sample V, where it represented more than 50% of the total GST activity. GST μ (pI 6.6) was present only in samples II, IV and VI, and its concentration in these samples was 45%, 3.5% and 40% respectively of total GST activity of liver. The apparent purity of GST ψ was established by urea/SDS/2-mercaptoethanol/polyacrylamide-gel electrophoresis, where GST ψ preparations showed the presence of a single protein band corresponding to an M_r of 26500 (results not shown). The apparent M_r of GST ψ was

determined to be about 53000 by gel filtration on a Sephadex G-100 column; a single coincident peak of enzyme activity and protein was observed. The presence of only one polypeptide spot on two-dimensional polyacrylamide-gel electrophoresis in the system of O'Farrell (1975) further confirmed the apparent homogeneity of GST ψ . In addition, quantitative automated sequence analysis showed only a single amino acid sequence, with no indication of significant contamination.

Amino acid composition and primary structure of GST ψ

The amino acid composition of GST ψ was determined, and the results are presented in Table 1. This composition was compared with that of GST π of human placenta and GST μ of human liver (Table 1). The difference index between GST ψ and π (Dao *et al.*, 1984) calculated by the method of Metzger *et al.* (1968) was found to be about 14.2. GST ψ was considerably higher in methionine, isoleucine and phenylalanine contents when compared with GST π , which was somewhat richer in leucine. The difference index between the amino acid compositions of GST ψ and GST μ (Alin *et al.*, 1985)

Table 1. Amino acid compositional analysis of human liver anionic GST ψ and comparison with GST μ of human liver and GST π of human placenta

The results are based on a subunit M_r of 26 500 for GST ψ . Values represent means $\pm$ S.D. of 24 h, 48 h and 96 h hydrolyses. Nearest-integer values are given in parentheses. Abbreviation: N.D., not determined.

Amino acid	Content (residues/molecule of subunit)			
	GST ψ (pI 5.5)	GST π (pI 4.5)*	GST μ (pI 6.6)†	
Aspartic acid	23.9 $\pm$ 1.4	(24)	21	25
Threonine‡	8.2	(8)	9	7
Serine‡	10.2	(10)	11	16
Glutamic acid	25.4 $\pm$ 3.2	(25)	24	27
Proline	8.9 $\pm$ 0.6	(9)	11	16
Glycine	20.3 $\pm$ 2.1	(20)	19	22
Alanine	19.7 $\pm$ 1.8	(20)	14	12
Valine§	17.8	(18)	13	6
Half-cystine	N.D.		2	5
Methionine§	5.6 $\pm$ 0.6	(6)	1	5
Isoleucine§	16.7 $\pm$ 2.0	(17)	6	10
Leucine	25.4 $\pm$ 3.5	(25)	31–32	26
Tyrosine	9.6 $\pm$ 0.6	(10)	10	12–13
Phenylalanine	21.5 $\pm$ 0.9	(21–22)	8	12
Lysine	16.5 $\pm$ 1.4	(16–17)	11	23
Histidine	4.9 $\pm$ 0.04	(5)	4	7
Arginine	11.2 $\pm$ 0.4	(11)	8	12
Total	(245–247)		(203–204)	(243–244)

* Dao *et al.* (1984).

† Alin *et al.* (1985).

‡ Extrapolated to zero time of hydrolysis.

§ Value obtained at 96 h of hydrolysis.

was found to be about 14.5. Noticeable differences in the amino acid compositions of these two enzymes occurred in the amounts of proline, alanine, valine, histidine and phenylalanine. Five cationic GST of human liver are shown to have similar amino acid compositions (Kamisaka *et al.*, 1975; Y. C. Awasthi *et al.*, unpublished work). The difference index between GST ψ and α , β , γ , δ , and ϵ was calculated to be about 14.8, and the most significant differences were observed in the contents of aspartic acid, alanine, proline, histidine, phenylalanine and lysine in the cationic isoenzymes and GST ψ .

Amino acid sequence analysis of the *N*-terminal region of GST ψ was determined, and a single polypeptide sequence was obtained, as shown in Table 2. This amino acid sequence was found to be identical with the 23

Table 2. Quantitative automated sequence analysis of GST ψ of human liver

Results were obtained with a gas-phase microsequencer. Amino acid phenylthiohydantoins were quantified by h.p.l.c.

Edman cycle	Residue (pmol)		Edman cycle	Residue (pmol)	
0	–	(1600)*	16	Ile	(128)
1	Pro	(462)	17	Arg	(13)
2	Met	(430)	18	Leu	(152)
3	Ile	(520)	19	Leu	(219)
4	Leu	(477)	20	Leu	(226)
5	Gly	(399)	21	Glu	(89)
6	Tyr	(328)	22	Tyr	(248)
7	Trp	(234)	23	Thr	(62)
8	Asp	(175)	24	Asp	(227)
9	Ile	(288)	25	His	(65)
10	Arg	(170)	26	Asp	(262)
11	Gly	(219)	27	Tyr	(192)
12	Leu	(271)	28	Leu	(231)
13	Ala	(285)	29	Gly	(121)
14	His	(54)	30	Lys	(234)
15	Ala	(232)			

* Initial pmol applied to the sequencer.

residues reported for GST μ , pI 6.6 (Alin *et al.*, 1985). Some striking *N*-terminal-region sequence homologies have been reported in GST isoenzymes, not only in the same species but also between human and rodents (Mannervik *et al.*, 1985). Although GST ψ appears to be similar to GST μ , it demonstrates some important differences. These two enzymes differ in their observed pI value by 1.1 pH unit, which is unlikely to be due to the difference in purification protocol. The pI of GST ψ was repeatedly found to be 5.5 ± 0.08 ($n = 6$), even when it was purified by the protocol for purification of GST μ reported by Warholm *et al.* (1983). In addition, the amino acid compositional difference between GST ψ and GST μ also indicates that the primary structures of GST ψ and GST μ are somewhat dissimilar.

Primary-structure comparison of the *N*-terminal region of GST ψ with that of other human and rat GST isoenzymes

The amino acid sequence of the *N*-terminal region of human liver GST ψ (Table 2) was compared with that of GST π of human placenta (Dao *et al.*, 1984), GST μ (Alin *et al.*, 1985) of human liver, and GST B, GST X (Frey *et al.*, 1983) and GST A of rat liver (Ding *et al.*, 1985), and the results are summarized in Table 3. This

Table 3. Comparison of residue identities between GST ψ and other reported GST isoenzymes

		Identities (%)				
		1	2	3	4	5
Rat liver GST A	1		68	33	30	77
Rat liver GST X	2	13/19		28	35	84
Rat liver GST B	3	6/18	5/18		28	28
Human placenta GST π	4	9/30	7/20	5/18		27
Human liver GST ψ	5	23/30	16/19	5/18	8/30	

No. of identities

Fig. 2. Comparison of the N-terminal-region sequence and predicted secondary structure of GST ψ with those of other reported mammalian GST isoenzymes

The predicted secondary structures of GST isoenzymes are shown below the respective amino acid sequence: β , β -sheet; α , α -helix, R, random coil; T, β -turn. The amino acid sequences of GST isoenzymes were taken from: GST A, Ding *et al.* (1985), from cDNA sequence analysis; GST X, Frey *et al.* (1983); GST π , Dao *et al.* (1984); GST μ , Alin *et al.* (1985); GST ψ , the present study.

region of amino acid sequence of GST ψ was found to be fairly homologous to those of rat liver GST A and X. There was about a 77% residue identity between human GST ψ and GST A of rat liver, and about 84% residue identity between human GST ψ and GST X of rat liver (Table 3). Among the human enzymes, GST ψ and GST μ were identical in their first 23 amino acid residues. Less than 30% residue identity was observed between the *N*-terminal sequences of GST π of placenta, GST B of rat liver and GST ψ .

Prediction of secondary structure of GST ψ

Secondary structure of GST ψ and those of other GST isoenzymes of rat and human tissues were predicted by the method of Chou & Fasman (1978). The predicted secondary structures at the *N*-terminals of GST ψ and μ of human liver, GST π of human placenta, and GST B, A, and X of rat liver, are summarized in Fig. 2. The secondary-structure prediction for GST π of human placenta (Dao *et al.*, 1984) was modified slightly in the light of primary-structure data for other GST isoenzymes. The β -turn at residues 11–14 was changed, as shown in Fig. 2. This region has a very marginal turn potential, $P_t = 0.753 \times 10^{-4}$ (Chou & Fasman, 1978).

DISCUSSION

This study describes the isolation, characterization and partial *N*-terminal-region sequence analysis of human liver GST ψ , and compares it with other reported GST isoenzymes. The GST ψ isoenzyme appears to be closely related to the human liver GST μ described by Warholm *et al.* (1983), as evidenced by the complete identity in the first 23 amino acids of their *N*-terminal region (Fig. 2). Although this suggests that these two GST isoenzymes may be identical, observed differences in their pI and amino acid compositions suggest that these two isoenzymes are non-identical but probably closely related proteins. The non-identity of GST ψ and GST μ is further suggested by differences in some of their kinetic properties, some such prominent differences being as follows. (a) GST ψ did not express GSH peroxidase II activity towards cumene hydroperoxide (Singh *et al.*, 1985), whereas a small amount of this activity was expressed by GST μ (Warholm *et al.*, 1983). (b) With chlorodinitrobenzene as the electrophilic substrate, bromosulphophthalein inhibits GST μ non-competitively, with a K_i of $1 \mu\text{M}$, whereas GST ψ is inhibited competitively by bromosulphophthalein with respect to chlorodinitrobenzene, with a K_i of $9 \mu\text{M}$ (Y. C. Awasthi & S. V. Singh, unpublished work). (c) With chlorodinitrobenzene as the other substrate, cholic acid is a competitive inhibitor of GST μ , with a K_i of $40 \mu\text{M}$ (Warholm *et al.*, 1983), whereas GST ψ is inhibited non-competitively by this bile acid, with a K_i of $125 \mu\text{M}$ (Y. C. Awasthi & S. V. Singh, unpublished work) at saturating concentration of GSH.

On the basis of structural and kinetic similarities, Mannervik *et al.* (1985) have classified the mammalian GST isoenzymes into three groups. These similarities are observed in both inter- and intra-species isoenzymes. The present data suggest a close structural interrelation between GST ψ and GST A and X of rat liver and also between GST ψ and GST μ of human liver. Thus GST ψ seems to belong to the class μ of mammalian GST according to the classification of Mannervik *et al.* (1985).

Even though GST ψ of liver and GST π of placenta are both anionic isoenzymes, there is only about 27% residue identity between these two isoenzymes in their *N*-terminal region (Table 3). This suggests that the A'-type subunits of GST ψ are very different from the C-type of subunits present in the placental GST π (CC) (Dao *et al.*, 1984) and liver GST ω , which has a subunit composition of BC (Dao *et al.*, 1982; Singh *et al.*, 1985). Immunological studies (Singh *et al.*, 1985) indicate that the A'-subunit of GST ψ is structurally distinct from the A- and B-type subunits of cationic liver GST. This is also supported by our unsuccessful attempts to determine the sequence of A- and B-type subunits, indicating blocked *N*-terminals. These studies therefore provide further evidence (Singh *et al.*, 1985) that several structurally distinct types of subunits are present among GST isoenzymes of human liver.

Genetic studies based on electrophoretic patterns (Board, 1981; Strange *et al.*, 1984, 1985) and chromatofocusing profiles of GST isoenzymes (Strange *et al.*, 1985) in cytosol of human tissues indicate that various human GST isoenzymes are products of at least three autosomal loci, designated as GST1, GST2 and GST3. Occurrence of more GST loci, GST4, GST5 and GSTe, has also been suggested by Laisney *et al.* (1984). The near-neutral isoenzymes have been suggested to be the products of the GST1 locus. If GST ψ , characterized in the present study, and other near-neutral isoenzymes, GST μ (Warholm *et al.*, 1983), GST1 1 (pI 5.85), GST1 2 (pI 6.35), and the intra-allelic hybrid isoenzyme (GST1 1/GST1 2), pI 6.1, reported by Strange *et al.* (1984, 1985), are products of a single polymorphic locus, a very close structural interrelationship must exist among these isoenzymes. Unfortunately structural data for purified isoenzymes are available only for GST μ (Alin *et al.*, 1985). The present study demonstrates a close structural interrelationship between GST μ and ψ , as indicated by their identical *N*-terminal-region sequence up to 23 amino acid residues. However, it must be pointed out that the variance in amino acid compositions of GST μ and ψ is more than that would be expected between two isoenzymes associated with the same polymorphic locus. Further structural studies are therefore needed to establish interrelationship among GST μ and GST ψ . The difference in the pI of GST ψ (present study, pI 5.5) and GST1 1 (Strange *et al.*, 1984, 1985; pI 5.85) is rather small. The possibility of GST1 1 and GST ψ being identical appears to be inconsistent with the predicted frequency of GST1 1 (one out of eight subjects; Strange *et al.*, 1984, 1985). However, the possibility of GST ψ not being detected by electrophoresis in the samples where its concentration is low cannot be ruled out. Furthermore, owing to the very small number ($n = 6$) of samples used in the present study, a meaningful genetic analysis cannot be performed.

Significantly, the predicted secondary structure of GST ψ is strikingly similar to the secondary structures of other reported mammalian GST isoenzymes. Despite substantial differences in the amino acid sequence of some of these isoenzymes, all demonstrated conserved secondary structure at their *N*-termini. Typically, for all these proteins a β -sheet structure initiates the *N*-terminal region for about 10 residues of the sequence (Fig. 2). This is successively followed by either a long α -helix or a small portion of random coil, and then an α -helix. A β -turn

immediately follows and is centred approximately around residue 25. Importantly, the general uniformity of the secondary structure of this enzyme class strongly indicates that all of the enzymes shown in Fig. 2 probably evolved as a result of a gene-duplication mechanism followed by divergence, and it is unlikely that a convergent evolutionary mechanism could account for the occurrence of these isoenzymes.

This is an important consideration, since related enzyme activity and even structural similarities in enzyme catalytic functions do not necessarily indicate that any two enzymes are homologous. A good example of this is the evolutionary relationship of the chymotrypsinogen family of serine proteinases to the serine proteinase subtilisin BPN'. Although there is remarkable similarity in the structural arrangement of the catalytic residues (charge-relay system) between subtilisin BPN' and the chymotrypsinogen family of serine proteinases, three-dimensional X-ray-crystallographic analysis gave strong evidence that this observed similarity was due to a convergent form of evolution (Alden *et al.*, 1970).

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