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Characterization of the Normal αI-Antitrypsin Allele V_{munich}: A Variant Associated with a Unique Protein Isoelectric Focusing Pattern

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Summary

α1-Antitrypsin (α1AT), the major serum inhibitor of neutrophil elastase, is a highly polymorphic protein associated with isoelectric focusing (IEF) patterns typical for each variant. α1AT V_{munich}, a previously unreported normal α1AT variant, has a unique IEF banding pattern in which the 7 and 8 α1AT protein bands focus with the normal M-type 7 and 8 bands, despite the fact that the major fraction of the V_{munich} protein focuses in the "V" region of the IEF gel. To characterize the molecular basis of this variant and its unique IEF pattern, DNA sequence analysis of the coding exons of the V_{munich} α1AT gene was carried out using the polymerase chain reaction. The V_{munich} allele differed from the common normal M1(Val^{213}) α1AT allele by a single nucleotide substitution of cytosine for adenosine, with the resultant amino acid change Asp^2 GAT→Ala GCT. Inheritance of the allele was confirmed by family analysis using allele-specific amplification with the polymerase chain reaction. The Asp^2→Ala mutation explains the cathodal position of the V_{munich} protein on IEF, as there is a substitution of a negatively charged amino acid by a neutral one. It is important that, in the context that the normal M-type α1AT 7 and 8 bands on IEF analysis are composed of α1AT molecules in which the first five residues have been cleaved, the position of the V_{munich} mutation within the first five amino acids accounts for the V_{munich} 7 and 8 bands focusing with the normal M-type 7 and 8 bands, i.e., the α1AT proteins making up these bands do not contain the first five amino acids and thus cannot reflect the difference between V_{munich} and the normal M1(Val^{213}) α1AT protein.

Introduction

α-Antitrypsin (α1AT), a 52-kDa glycoprotein, is the major serum inhibitor of neutrophil elastase, a powerful serine protease capable of destroying most components of the extracellular matrix (Travis and Salvesen 1983; Bieth 1986). Almost all of the α1AT protein in serum is produced by hepatocytes as a 394-residue single-chain molecule with three complex asparaginyl-linked carbohydrate side chains (Carrell et al. 1982; Brantly et al. 1988; Cox 1989). The 12.2-kb α1AT gene is located on chromosome 14 at q31-32.2 and is composed of seven exons (I_A, I_B, I_C, and II–V) and six introns (Long et al. 1984; Rabin et al. 1986; Perlino et al. 1987). It is highly polymorphic, encoding for more than 75 different α1AT variants identified by either isoelectric focusing (IEF) of serum and/or sequence analysis (Brantly et al. 1988; Cox 1989; Crystal et al. 1989).

For a given variant, the IEF patterns reveal microheterogeneity consisting of two major bands (referred to as 4 and 6) and three minor bands (2, 7, and 8). For the common M-type "normal" α1AT variants, this microheterogeneity is attributed to the differences in the three carbohydrate side chains and to the length of the α1AT protein (see schematic; fig. 1) (Vaughan et al. 1982; Hercz 1985; Jeppsson et al. 1985). The major 4 band has two biantennary carbohydrate side chains and one triantennary side chain, whereas the major 6 band has three biantennary side chains. The more anodal, minor 2 band has two triantennary side

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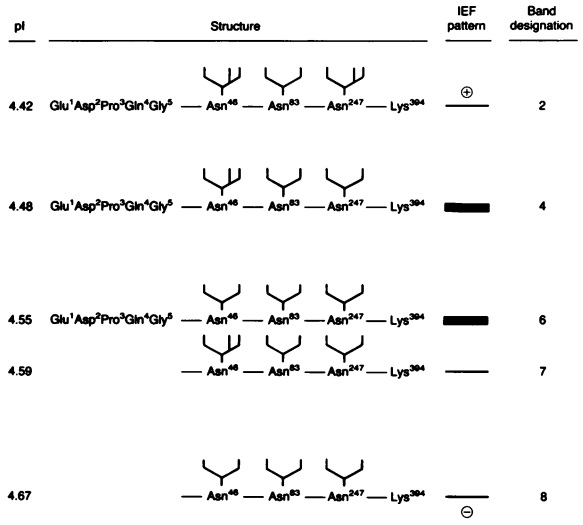

Figure 1 Microheterogeneity of serum IEF of normal M-type α1AT protein and structure of α1AT molecules corresponding to each band. The serum α1AT proteins associated with the homozygous inheritance of normal M-type α1AT gene demonstrate a microheterogeneity based on differences in carbohydrate side chains (indicated by vertical structures attached to Asn residues at positions 46, 83, and 247) and the loss of the five terminal amino acids $Glu^1Asp^2Pro^3Gln^4Gly^5$. At the left is the pI (isoelectric point) of each band. In the middle is the structure; note that there are two different carbohydrate side-chain types—biantennary and triantennary. The three forms focusing at the lower pH range have 394 amino acids, while the two forms focusing at the high pH range are missing the five N-terminal residues. At the right is a schematic both of the focusing pattern and of a band designation with the anode (+) at the top and the cathode (−) at the bottom. Bands 4 and 6 are the major bands, with 2, 7, and 8 bands being less abundant (figure is based on Jeppsson et al. 1985, fig. 4).

chains and one biantennary side chain. The 7 and 8 bands are identical to the 4 and 6 bands, respectively, except that they do not contain the first five amino acids of the mature α1AT protein, thought to be caused by proteolytic cleavage by unknown mechanisms. These N-terminal five amino acids include the sequence $Glu^1Asp^2Pro^3Gln^4Gly^5$—two negatively charged and three neutral amino acids—thus explaining the cathodal position of these minor α1AT bands relative to the major 4 and 6 bands.

The purpose of the present study is to characterize a newly identified α1AT variant, V_{munich}, an α1AT variant with unique IEF characteristics in which the microheterogeneity pattern does not fit the "classic" pattern of the relative positions of the various bands defined for the common normal M variants. It is interesting that analysis of the coding exons of the V_{munich} gene yielded an explanation for this unique banding pattern.

Methods

Study Population

Three individuals—the index case and both parents—of a family carrying the α1AT V_{munich} allele were available for analysis. The α1AT phenotype was determined by a combination of IEF and α1AT serum levels (Cox et al. 1980; Fagerhol and Cox 1981). IEF was performed with carrier pharmalytes on flat-bed, 0.5-mm polyacrylamide gels according to a method described elsewhere (Weidinger et al. 1985). To determine that V_{munich} focused in the "V" range, the phenotypes evaluated in parallel as standards included M1V (the "V" allele is different from "V_{munich}"; see Fagerhol and Laurell 1967), M1S, M3S, and $M2P_{saint\ louis}$ (Pierce and Eradio 1981). In addition, enhancement of the α1AT protein bands was achieved by an immunofixation print utilizing a rabbit anti-human α1AT antibody (Accurate Chemical and Scientific) followed by binding with gold conjugate (Biorad) (Boutin et al. 1985). α1AT serum levels were measured by nephelometry (Behring Nephelometer-Analyzer). To avoid confusion with commonly used standards that overestimate α1AT levels, values are expressed in micromolar units (normal levels 20–48 μM) and are based on a true laboratory standard. To convert these values to standards commonly used in clinical studies (in mg/dl), multiply by 7.32 (Wewers et al. 1987).

Sequence Analysis of the V_{munich} Allele

Genomic DNA was extracted from peripheral white blood cells (Jeffreys and Flavell 1977). With this DNA as a template, 200 bp 5′ to exon I_C (the region of the major liver promoter) and exons I_C–V, together with the flanking intron sequences of the α1AT genes of the index case, were amplified by the polymerase chain reaction (PCR) using oligonucleotide primers based on the normal α1AT sequence, *Thermus aquaticus* DNA polymerase (Cetus), and a thermal cycler (Perkin-Elmer Cetus) under recommended conditions (Saiki et al. 1985, 1988) The resulting fragments were purified by 1% agarose gel electrophoresis followed by electroelution, phenol-chloroform extraction, and ethanol precipitation. A portion (0.5 ng) of this purified amplified DNA was used as a template for single-stranded DNA generation using the same oligonucleotide primers as the initial amplification but utilizing the unequal primer concentration method (Gyllensten and Erlich 1988). The resultant single-strand DNA was sequenced by the dideoxy chain termination method using T7 polymer-

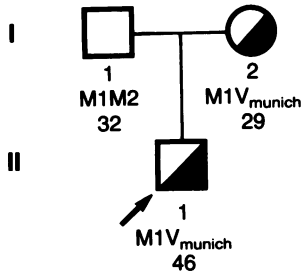

Figure 2 Pedigree of a family carrying the α1AT V_{munich} allele. Generations I and II are shown. Below each family member is a number for identification, followed by the α1AT phenotype and α1AT serum levels in micromolar units (see Methods for explanation of α1AT-level measurement). The index case is indicated by the arrow.

ase (Sequenase, United States Biochemical) and was analyzed by 6% polyacrylamide, 8 M urea gel electrophoresis and autoradiography (Sanger et al. 1977).

Inheritance of the V_{munich} Allele

To demonstrate inheritance of the V_{munich} allele, allele-specific amplification with PCR was used to analyze genomic DNA of each family member (Newton et al. 1989; Okayama et al. 1989). Allele-specific amplification primers were constructed such that their 3′ terminal base corresponded either to the normal base at the site of the V_{munich} mutation or, alternatively, to the V_{munich} mutation. In combination with a common

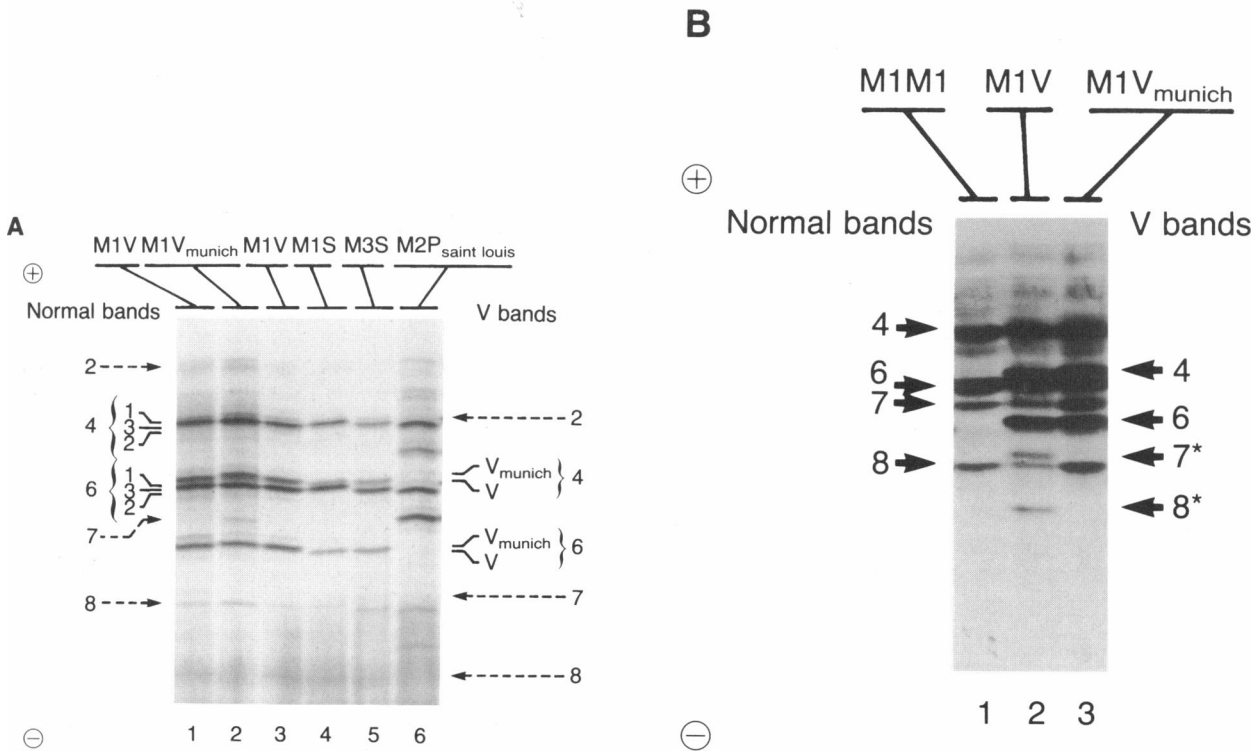

Figure 3 Characterization of α1AT V_{munich} by IEF. *A*, IEF at pH 4.2–4.9. The anode (+) is at the top, and the cathode (−) is at the bottom. The five normal α1AT bands (2, 4, 6, 7, and 8) are shown on the left. For the major 4 and 6 bands, the positions of the M1-type, M3-type, and M2-type α1AT alleles are indicated. On the right, the five corresponding V α1AT bands are identified. For the major 4 and 6 bands, both the V_{munich} and V bands are shown, indicating the small difference in position of the major 4 and 6 bands of these two variants. At the top of each lane is shown the α1AT phenotype: lane 1, M1V; lane 2, M1V_{munich} (index case); lane 3, M1V; lane 4, M1S; lane 5, M3S; lane 6, M2$P_{saint\ louis}$. *B*, IEF with immunofixation by rabbit anti-human α1AT antibody, followed by binding with gold conjugate. The anode (+) is at the top, and the cathode (−) is at the bottom. Normal α1AT bands (4, 6, 7, and 8) are shown on the left, and the V α1AT bands are shown on the right (4, 6, 7, and 8) showing absence of V_{munich} 7 and 8 bands in the predicted position (indicated by 7* and 8*). At the top of each lane is the α1AT phenotype: lane 1, M1M1; lane 2, M1V; lane 3, M1V_{munich}.

distal primer 503 bases 3′ to the V_{munich} mutation site, amplification occurs only if the allele-specific primer has complementarity at its 3′ end to the template DNA. Resultant amplification products were analyzed by 1% agarose gel with ethidium bromide staining.

Results

Identification of the α IAT V_{munich} Variant

Family analysis revealed that two individuals—the index case (II_1) and the mother (I_2)—had the α1AT V_{munich} protein (fig. 2). Both of these individuals had α1AT serum levels in the normal range, consistent with V_{munich} being an α1AT allele associated with normal α1AT synthesis and secretion. IEF analysis showed that the V_{munich} variant was in the "V" range, just anodal to the V type α1AT but significantly cathodal to the $P_{saint\ louis}$ variant (fig. 3*A*). In addition, IEF showed that the M1V_{munich} index case appeared to lack V_{munich} 7 and 8 α1AT protein bands when compared with those of the M1V individual. The absence of V_{munich} 7 and 8 bands was more apparent with immunofixation with a rabbit anti-human α1AT antibody performed to highlight the α1AT protein bands (fig. 3*B*).

Elucidation of the Molecular Basis of the V_{munich} Variant

DNA sequence analysis of the index case revealed that the M1 allele of this individual was an M1(Val^{213}) allele and that the V_{munich} allele differed from the normal M1(Val^{213}) allele by a single nucleotide mutation of adenosine to cytosine in the codon for amino acid 2 of the mature α1AT protein (fig. 4). In this regard, while the normal M1(Val^{213}) homozygote control had the sequence Asp^2 GAT, the M1(Val^{213})V_{munich} heterozygote individual had both Asp^2 GAT and Ala^2 GCT, showing that the V_{munich} allele coded for alanine at amino acid position 2. This mutation explains two interesting features of the V_{munich} variant: (1) the cathodal position of the major V_{munich} 4 and 6 α1AT protein bands on IEF and (2) the reason for absence of the V_{munich} 7 and 8 bands in the predicted position (see Discussion).

Confirmation of Inheritance of the V_{munich} Allele

Analysis of both the genomic DNA of each family member and an M1(Val^{213}) homozygote control by allele-specific amplification with PCR showed that the V_{munich} allele of the index case was inherited in a codominant fashion from the mother (fig. 5). Using the allele-specific amplification primer with its 3′ terminus based on the normal sequence at the V_{munich} mutation site, (M_VM) in combination with a common distal primer, amplification of the DNA of the normal [M1(Val^{213})] homozygote control as well as of all family members was observed, consistent with each having at least one normal allele (fig. 5, lanes 1, 3, 5, and 7). Furthermore, the allele-specific amplification primer with its 3′ end complementary to the V_{munich} mutation (fig. 5, VM) in combination with the common distal primer did not amplify the DNA of either the normal control or the father (fig. 5, lanes 2 and 4) but did amplify the DNA both of the index case and of the mother

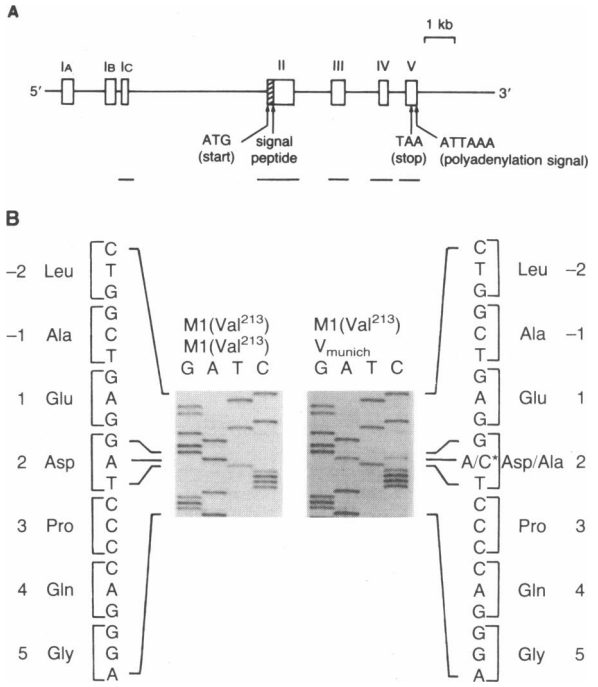

Figure 4 DNA sequence analysis of M1V_{munich} index case. *A*, Schematic of α1AT gene, to delineate areas sequenced. The seven exons (I_A, II_B, I_C, and II–V) and six introns are shown. Shading in exon II indicates the signal peptide. The start (ATG) and stop (TAA) codons are shown, as is the polyadenylation signal. Areas sequenced are underlined. *B*, Autoradiograms of sequencing gels. Shown on the left is an M1(Val^{213}) homozygote control, and shown on the right is the M1(Val^{213})V_{munich} index case. In each, the four lanes representing the nucleotides G, A, T, and C, respectively, are shown. The exon II nucleotide sequences are indicated, as are the corresponding sequences for amino acids −2 to 5 (amino acids −1 and −2 are part of the leader peptide of α1AT). The M1V_{munich} DNA differs from the M1(Val^{213}) homozygote by having both the normal adenosine and cytosine at the second nucleotide (indicated by the asterisk) of the codon for amino acid 2, showing that the index case is both Asp and Ala at this codon. Therefore, the V_{munich} allele differs from the M1(Val^{213}) allele by Asp^2 GAT→Ala^2 GCT. The rest of the sequence analysis of the M1V_{munich} DNA revealed no other mutations compared with the normal M1(Val^{213}) allele.

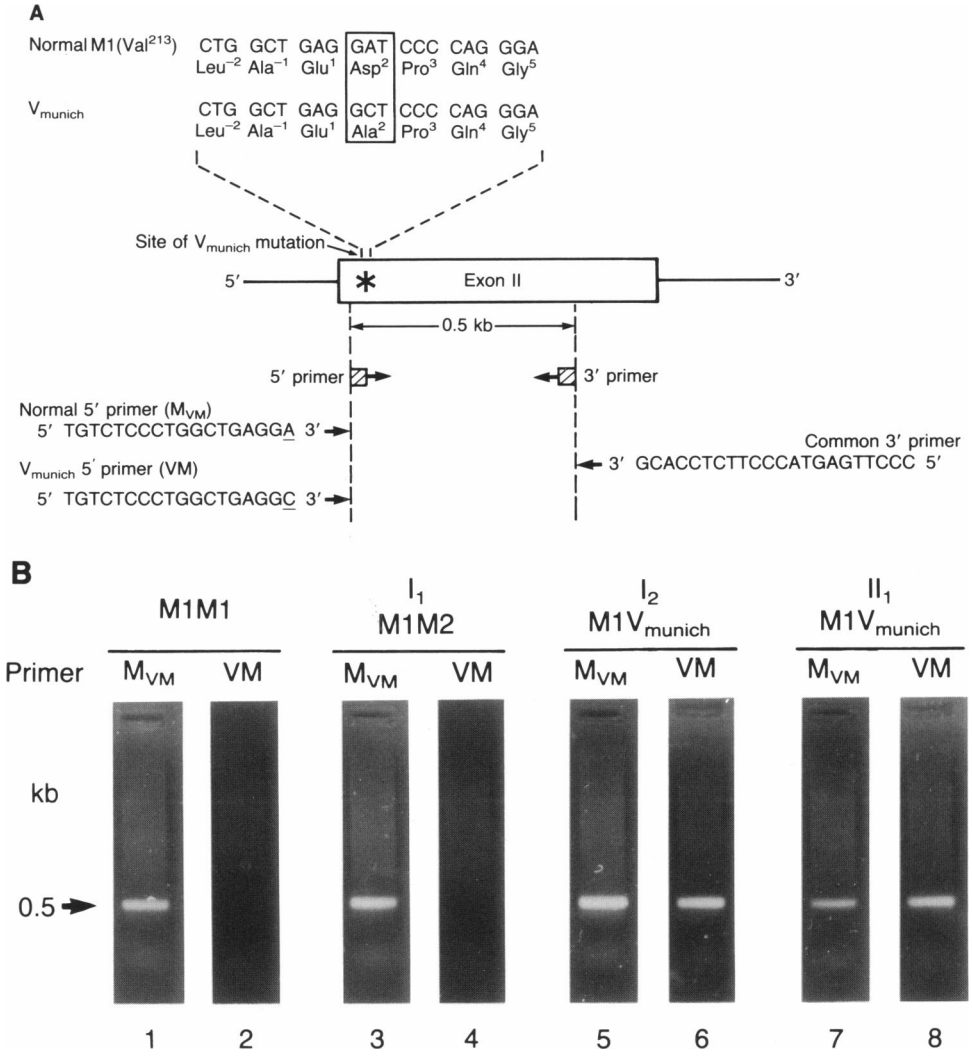

Figure 5 Allele-specific amplification with polymerase chain reaction to demonstrate inheritance of α1AT V_{munich} allele. *A*, Schematic showing the nucleotide and amino acid sequence of both the normal M1(Val^{213}) allele and the V_{munich} allele in the region of the V_{munich} mutation in the codon for amino acid 2 in exon II (boxed area). The diagram of exon II indicates the site of V_{munich} mutation. Below this on the left are both the sequence of the normal allele-specific 5′ primer (M_{VM}) and the sequence of the V_{munich} allele-specific primer (VM), with the mutational difference underlined. In combination with the common 3′ distal primer on the right, a 0.5-kb amplification product will be generated if the 3′ terminus of the 5′ primer is complementary to the template DNA. *B*, Result of allele-specific amplification for an M1M1 control and for each family member as indicated at the top of the figure. Above each lane is the allele-specific primer used, and on the left is indicated the size (0.5 kb) of the amplification product. The M1M1 control amplifies only with primer M_{VM}, the normal primer at the V_{munich} mutation site (lanes 1 and 2), as does family member I_1, an M1M2 heterozygote (lanes 3 and 4). Family members I_2 and II_1 amplify with both primer M_{VM} and primer VM (lanes 5 and 6 and lanes 7 and 8, respectively), consistent with each being an M1V_{munich} heterozygote and showing that inheritance of the V_{munich} allele in family member II_1 is from family member I_2.

(fig. 5, lanes 6 and 8), confirming that they both carry the V_{munich} mutation and that the index case had inherited the V_{munich} allele from the mother.

Discussion

IEF plays a major role in the categorization of the polymorphic protein α1AT. All previously described normal and deficient α1AT variants categorized by IEF have conformed to the typical pattern of two major 4 and 6 protein bands and three minor bands, i.e., the anodal 2 band and the two cathodal 7 and 8 bands (Vaughan et al. 1982; Hercz 1985; Jeppsson et al. 1985; Brantly et al. 1988; Cox 1989). In the present study we de-

scribe V_{munich}, a normal level α1AT variant whose departure from the typical IEF pattern can be explained by its molecular basis.

DNA sequence analysis revealed that V_{munich} differs from the normal M1(Val^{213}) α1AT allele by a single nucleotide substitution of adenosine by cytidine, causing an amino acid change, Asp^2 GAT→Ala GCT. The substitution of a negatively charged aspartic acid by a neutral alanine, creating a more positively charged protein, accounts for the cathodal position of the major 4 and 6 bands of the V_{munich} variant. Furthermore, the amino acid substitution within the first five amino acids of the V_{munich} protein explains why, in an M1(Val^{213}) V_{munich} heterozygote, V_{munich} 7 and 8 bands are missing in the expected position. As the only difference between V_{munich} and M1(Val^{213}) is within the first five N-terminal amino acids, and since the α1AT 7 and 8 bands are composed of molecules missing the first five amino acids of the mature α1AT protein, removal of this one difference between the V_{munich} and M1(Val^{213}) proteins causes the V_{munich} 7 and 8 bands to focus with the normal M-type 7 and 8 bands (fig. 6). Whether the α1AT protein comprising the 7 and 8 bands on IEF is functional in vivo is unknown. Further, it is not known what causes the cleavage of the mature α1AT molecule between the fifth and sixth amino acids to form the 7 and 8 bands (Hercz 1985).

Although charge and IEF pattern of the major V_{munich} protein is not very different from that of the deficiency S type variant, V_{munich} is associated with normal α1AT levels. This is consistent with the position of the amino acid substitution within the three-dimensional structure of the α1AT molecule. Whereas the S variant substitution is within an a helix (Glu^{264}→Val) and is thought to disrupt a salt bridge between this residue and residue 387, the V_{munich} mutation is not within any defined critical area of the mature α1AT protein (Owen and Carrell 1976; Loebermann et al. 1984).

Because of the highly polymorphic nature of α1AT, it is possible that further interesting variants affecting the pattern of IEF microheterogeneity will be found. In this regard, careful analysis of the changes in pattern may allow deduction of the likely area of mutation either within the coding sequence for the first five amino acids of the α1AT molecule or in the codons for the three asparagine residues (Asn 46, Asn 83, and Asn 247) to which the carbohydrate side chains are attached (Carrell and Owen 1979).

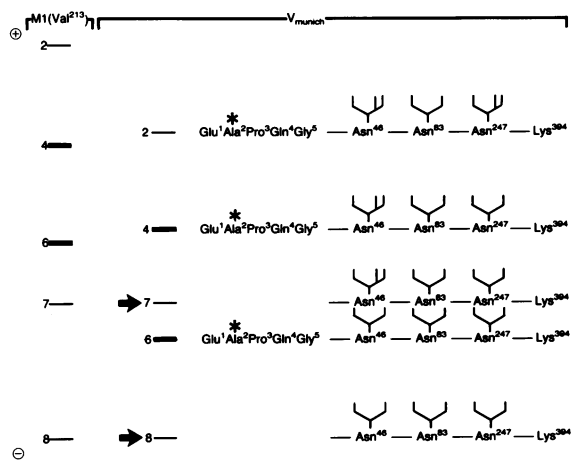

Figure 6 Basis of α1AT V_{munich} IEF pattern. On the left is the IEF pattern for the M1(Val^{213}) protein, with anode (+) at the top and cathode (−) at the bottom, showing the two major bands (4 and 6) and three minor bands (2, 7, and 8). On the right, the V_{munich} α1AT is shown to have these same bands, but the V_{munich} 7 and 8 bands focus with the normal M-type 7 and 8 bands (arrows). This is explained (far right) by the only amino acid difference between M1(Val^{213}) and V_{munich} being in the first five amino acids (residue 2, indicated by an asterisk), which are cleaved to form the 7 and 8 α1AT bands, and thus the proteins forming the 7 and 8 bands for M1(Val^{213}) and V_{munich} α1AT are identical.

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