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Phylogenetic Relations of Humans and African Apes from DNA Sequences in the ψη-Globin Region

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Sequences from the upstream and downstream flanking DNA regions of the ψη-globin locus in *Pan troglodytes* (common chimpanzee), *Gorilla gorilla* (gorilla), and *Pongo pygmaeus* (orangutan, the closest living relative to *Homo*, *Pan*, and *Gorilla*) provided further data for evaluating the phylogenetic relations of humans and African apes. These newly sequenced orthologs [an additional 4.9 kilobase pairs (kbp) for each species] were combined with published ψη-gene sequences and then compared to the same orthologous stretch (a continuous 7.1-kbp region) available for humans. Phylogenetic analysis of these nucleotide sequences by the parsimony method indicated (i) that human and chimpanzee are more closely related to each other than either is to gorilla and (ii) that the slowdown in the rate of sequence evolution evident in higher primates is especially pronounced in humans. These results indicate that features (for example, knuckle-walking) unique to African apes (but not to humans) are primitive and that even local molecular clocks should be applied with caution.

THE THREE CONTEMPORARY SPECIES of African apes [common chimpanzee (*Pan troglodytes*), pygmy chimpanzee (*Pan paniscus*), and gorilla (*Gorilla gorilla*)] are the closest living relatives of human (*Homo sapiens*). These species collectively represent a natural monophyletic group, which in turn is closely related to the orangutan (*Pongo pygmaeus*) of southeastern Asia. This genealogical arrangement is no longer in serious dispute, but the phylogenetic relations among humans and African apes are still open to debate (*1, 2*). Questions concerning human origins (man's closest living relative or relatives) remain despite earlier efforts to more fully resolve their relations from both molecular and anatomical evidence (*3*).

The β-globin gene family in primates has been well characterized in terms of its structure and evolution (*4*). In humans and great apes, the ψη-locus is one of six β-related globin genes linked 5′-ε-(embryonic)-^{G}γ-^{A}γ(fetal)-ψη (inactive)-δ-β (adult)-3′ (*5, 6*). By means of clones previously described (*7*), the 5′ and 3′ noncoding flanking regions of the ψη-globin gene were sequenced from common chimpanzee, gorilla, and orangutan [the closest living relative to *Homo*, *Pan*, and *Gorilla* (*1, 2*)], in an attempt to resolve the phylogenetic relations of humans and African apes. These newly sequenced orthologs (an additional 4.9 kbp for each species) were combined with published ψη-gene sequences (*5, 8*) and then compared to the same orthologous region available for humans (*9*). The four aligned sequences spanning nearly 7.1 kbp of noncoding DNA constituted the longest continuous stretch of orthologous DNA currently available for humans and great apes (Fig. 1). Furthermore, the nucleotide sequence from a second human allele of the ψη-globin locus (2.2 kbp) was included in the study (*5, 8*). This sequence allowed us to consider the significance of intraspecific polymorphism in reconstructing phylogenies (*10, 11*).

Pairwise comparisons among the ψη-locus and ψη-flanking sequences of human, chimpanzee, and gorilla reveal that these orthologs have diverged very little from one another (Table 1). The aligned DNA sequences of these three varied by only 1.6 to 2.1%, with human and chimpanzee being most alike. On average, the nucleotide sequence of orangutan differed from those of the other three by 3.6%. These values conform closely to the divergence estimates reported from DNA-DNA hybridization of single-copy DNA (*12*) and from nucleotide sequencing of other noncoding genomic regions (*8, 13–15*).

Only three dichotomous branching patterns are possible for human, chimpanzee, and gorilla (Fig. 2). Phylogenetic analysis of these possibilities by the parsimony method established that the tree with the human and chimpanzee clade is more parsimonious than its two alternatives (*Gorilla* grouping with *Pan* or *Homo* first) by five and six extra mutations, respectively (*16, 17*). Human and chimpanzee are united in the most parsimonious phylogeny by eight putative synapomorphies [shared derived features (*18*)] that represent two transitions (positions 1338 and 4473), three transversions (positions 560, 5480, and 6971), and three gap events (two deletions at positions 1287 and 3054 to 3057 and one insertion in a homonucleotide repeat at position 3272). The two less parsimonious solutions (gorilla grouping with chimpanzee or human first) are supported by only three base substitutions [one transversion (position 5153) and two transitions (positions 5156 and 6808)] and two transitions (positions 34 and 6368), respectively.

Thus, the 7.1-kbp sequences from the ψη-globin region demonstrate that human and chimpanzee are more closely related to each other than either is to gorilla. This arrangement is most heavily supported from independent sources by the DNA-DNA hybridization data of Sibley and Ahlquist [(*12*), and discussion (*19*)]. Other data sets in agreement with this phylogeny include α- and γ-globin protein sequences (*20*), and, to a somewhat lesser extent, the mitochondrial DNA sequences of Brown *et al.* (*21*) [as analyzed by Andrews (*2*) and Hasegawa and Yano (*22*)].

The time of divergence for the initial separation of human, chimpanzee, and gorilla is usually placed somewhere between 5 million and 10 million years ago (*23, 24*). Rates of ψη-globin evolution, as calculated with these dates and the branch lengths of the most parsimonious phylogeny (Fig. 2),

Table 1. Pairwise comparisons of the 7.1-kbp ψη-globin sequences for *Homo sapiens* (HSA), *Pan troglodytes* (PTR), *Gorilla gorilla* (GGO), and *Pongo pygmaeus* (PPY). The following abbreviations regarding base substitutions are used: BP, base positions under comparison; TS, transitions; TV, transversions; and TS/TV, ratio of transitions and transversions. Gaps refer to both insertion and deletion events (*17*). Pairwise percentages of divergences are calculated by the equation: [(TS + TV + gaps)/(BP + gaps)] × 100%.

Species comparison	Substitutions				Gaps	% Divergence
	BP	TS	TV	TS/TV		
HSA/PTR	6974	72	29	2.5	12	1.6
HSA/GGO	6984	77	23	3.3	17	1.7
HSA/PPY	6913	139	61	2.3	33	3.4
PTR/GGO	7020	90	36	2.5	19	2.1
PTR/PPY	6929	151	77	2.0	38	3.8
GGO/PPY	6945	159	66	2.4	33	3.7

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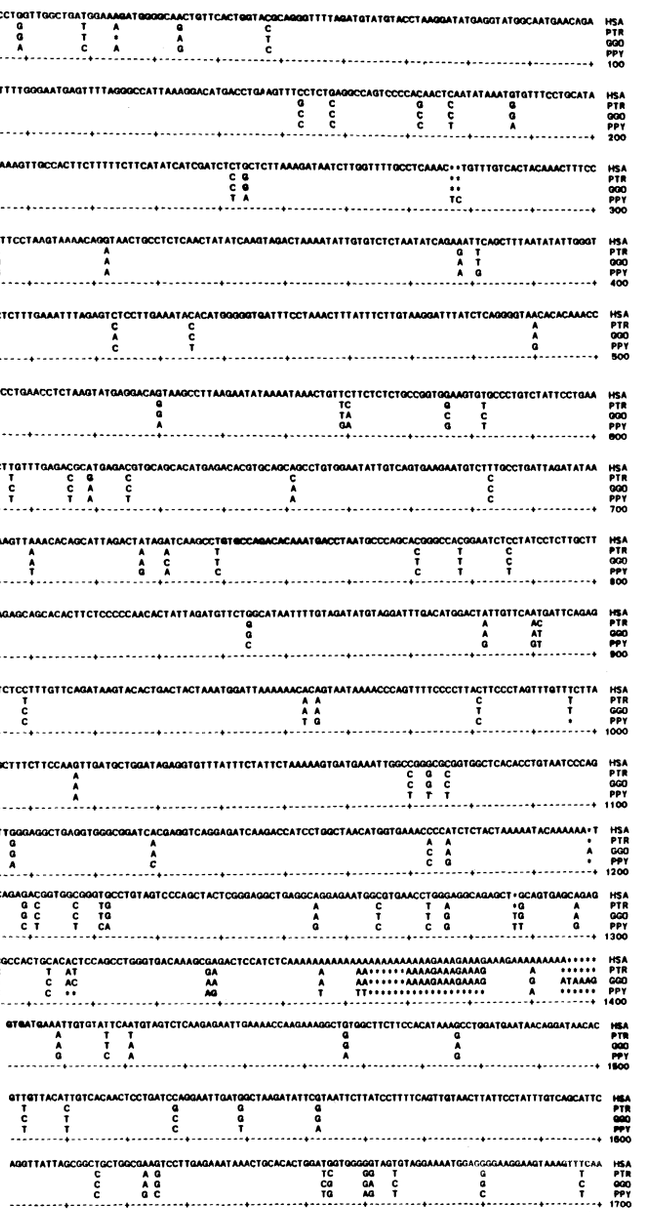
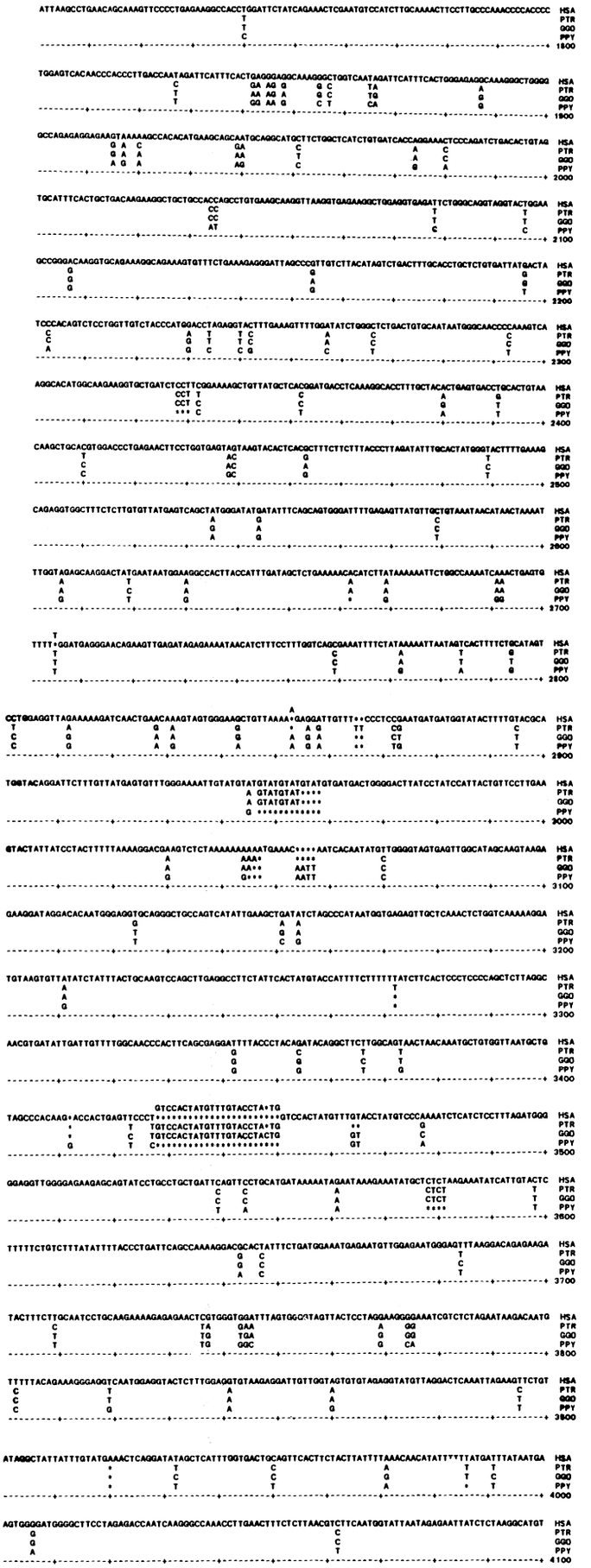
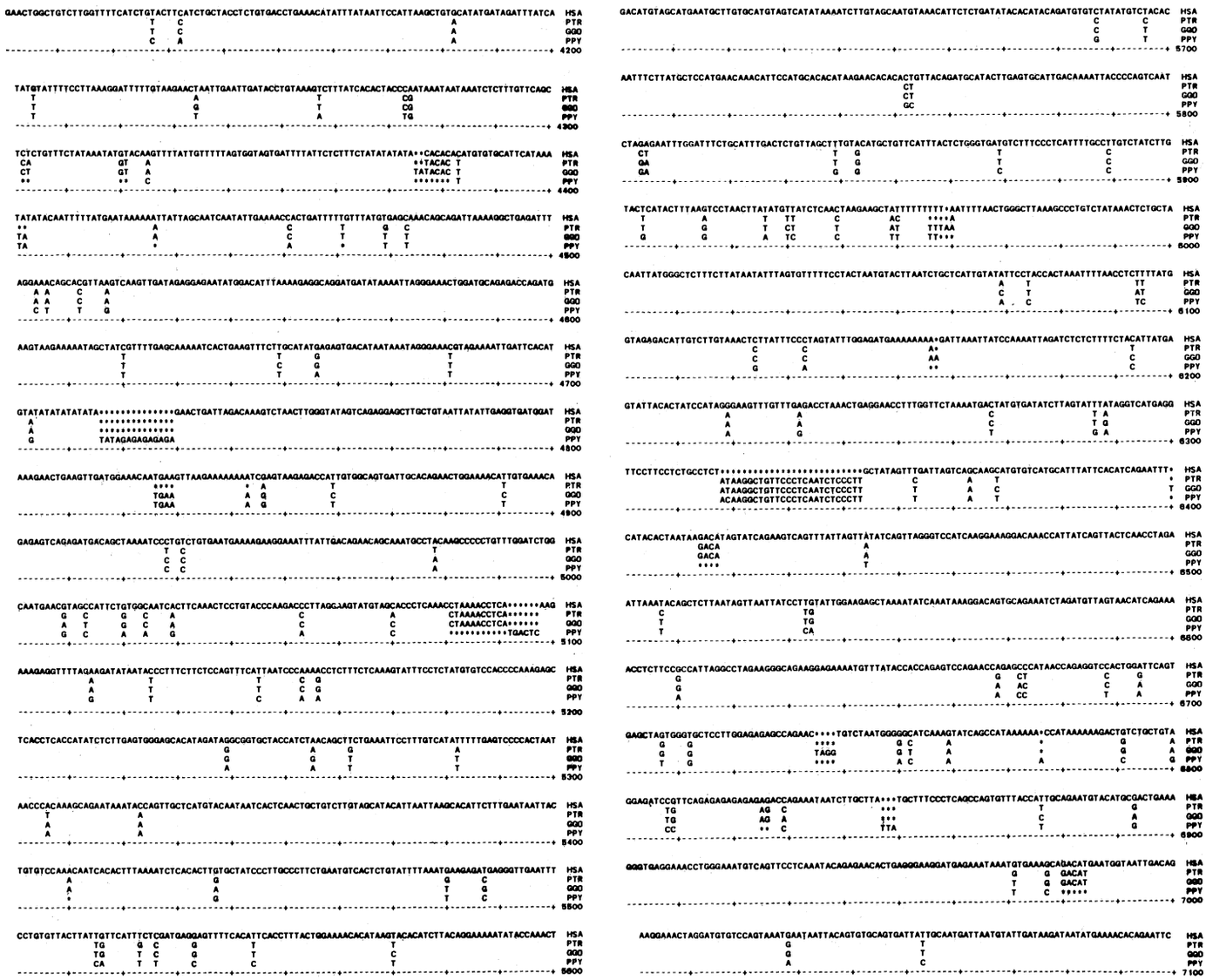
Fig. 1. Aligned DNA sequences of the 7.1-kbp ψη-globin region from *Homo sapiens* (HSA), *Pan troglodytes* (PTR), *Gorilla gorilla* (GGO), and *Pongo pygmaeus* (PPY). The complete nucleotide sequence of HSA is shown, whereas the corresponding bases of the other three are indicated only at variable sites. The nucleotide numbering system used in the diagram corresponds to the overall alignment recognized for these sequences. Asterisks in the alignment refer to gaps that are used to maximize identity among the four (*6, 30*). The ψη-gene sequences for PTR, GGO, and PPY (positions 1622 to 3782) are from Koop *et al.* (*8*). The complete 7.1-kbp sequence of HSA is from Collins and Weissman (*9*). The same HSA clone used by them (p ψβ 101-7.2) was resequenced to ensure accuracy, and corrections have been made at several positions (*31*). Corrections have also been made at several positions in the gene sequences of PTR and GGO (*31*). All four 7.1-kbp sequences share an Alu repeat element at the same orthologous location [positions 1068 to 1400 (*15*)]. The locations of exons, introns, promoter sequences, and other regulatory and structural elements have been discussed elsewhere (*5, 8*). A second human sequence of the ψη-globin locus (positions 1622 to 3782) is known (*5, 8*). This alternative allele varies from the corrected HSA sequence of Collins and Weissman (*9*) by three gap differences [at positions 2701 to 2705 (five T instead of four); 2849 to 2853 (five A rather than four); and 3428 to 3472 (presence versus absence of two direct contiguous repeats)]. These polymorphisms relative to the second allele are indicated in the figure above the ψη-gene sequence of HSA.







reveal that the human lineage has evolved about 30 to 40% slower than the chimpanzee and gorilla lines since their last common ancestor (0.7×10^{-9} to 1.5×10^{-9} versus 1.1×10^{-9} to 2.3×10^{-9} and 1.1×10^{-9} to 2.1×10^{-9} mutations per site-year, respectively). This slowdown in the rate is not dependent on the relatively few mutations with multiple parsimonious solutions, since the same trend is observed when only the unambiguous changes are considered [the branch lengths in brackets (Fig. 2)]. Thus, the rate of sequence evolution in human remains about 60 to 70% of that for chimpanzee or gorilla, whether or not the ambiguous changes are counted. These differences indicate that the human has experienced an even greater slowdown in the rate than apes and other primates (*8, 25*). Slower human rates appear to be characteristic of both nuclear and mitochondrial sequences (*25, 26*) and may be due to a longer generation time or an improved DNA repair mechanism, or both, in humans (*27*).

The ψη-globin sequence data therefore provide strong evidence for the following conclusions: (i) that human and chimpanzee are more closely related to each other than either is to gorilla and (ii) that the slowdown in the rate of nucleotide evolution evident in higher primates is especially pronounced in humans. One implication of the first conclusion is that attributes unique to chimpanzee and gorilla are probably primitive (rather than derived) features of humans and African apes. Thus, the human lineage most likely originated from an ancestor with African ape features [that is, locomotion by knuckle-walking (*24, 28*)]. An important implication of the second conclusion is that even local molecular clocks should be applied with caution (*1, 8*). Rate differences of 30 to 40% (as between humans and the African apes) must be identified beforehand, if more precise estimates of divergence times are to be made with sequence data (*29*).

The short common stem between human and chimpanzee [eight total mutations along this internode (Fig. 2)] indicates that the two branching points of the most parsimonious phylogeny occurred close in time [within 500,000 to 1,000,000 years of each other (*29*)]. This conclusion raises the question of error during the reconstruction of phylogeny by intraspecific polymorphism (*10, 13*). Errors in depicting the branching order of closely related species can occur by the random loss of alleles during the evolutionary descent of a group from a polymorphic ancestor. Furthermore, a different type of random error is illustrated by the two human alleles of the ψη-globin locus (Fig. 1). These two alleles represent a recent (rather than ancient) polymorphism that varies by only three gap differences (0.14%). However, two of these differences occur at

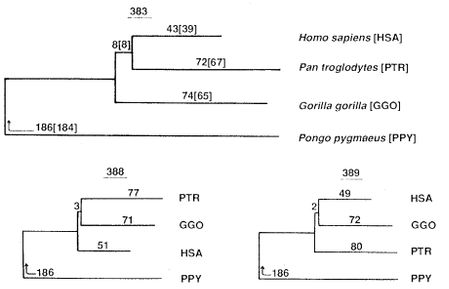


Fig. 2. The three possible dichotomous arrangements of *Homo sapiens* (HSA), *Pan troglodytes* (PTR), and *Gorilla gorilla* (GGO), as rooted against the closely related reference (*2, 32*), *Pongo pygmaeus* (PPY). For diagrammatic purposes only, the branch leading to PPY was drawn in each case as though it were rooted in the middle of the longest path between two species. The phylogeny shown on top constitutes the most parsimonious arrangement of the sequence data [total number of substitutions and insertions or deletions = 383 (*33*)]. The two less parsimonious alternatives to the lower-left and lower-right require 388 and 389 mutations, respectively. Branch lengths for the three phylogenies are drawn proportional to the total numbers of base substitutions and gap events associated with each link. Those character-state changes with more than one equally parsimonious solution are assigned to terminal branches, rather than to internal internodes (*34*). Branch lengths based only on unambiguous changes (those with one parsimonious solution relative to the HSA and PTR arrangement) are indicated in brackets for the most parsimonious phylogeny (*35*).

sites that also vary among the great apes (positions 2853 and 3428 to 3450). When both human alleles are considered together, the same number of mutations (five) is added to each of the three possible phylogenies (Fig. 2). Thus, the mutations at these polymorphic sites do not influence which of the three arrangements is most parsimonious. However, if only the human ψη-gene sequence of Collins and Weissman (*9*) is considered, two extra events [a homonucleotide deletion (position 2853) and a duplication resulting in two direct contiguous repeats (positions 3428 to 3450)] would be added in support of the human-chimpanzee and chimpanzee-gorilla solutions, respectively. In contrast, no such changes would be added if only the ψη-gene sequence reported by Chang and Slightom (*5*) is used. The best way to reduce the problems posed by intraspecific variation would be to acquire more nucleotide sequences from different unlinked regions and individuals. By obtaining new sequences from both humans and great apes, a better understanding of intraspecific polymorphism and its phylogenetic significance will be possible.

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35. The ambiguity mentioned in (*34*) refers to the process of selecting one phylogeny over the others. In contrast, the ambiguity referred to here concerns whether a mutation can be unequivocally assigned to one branch of the most parsimonious arrangement. For example, the single base substitution required at site 1317 can be uniquely assigned to the chimpanzee branch of the most parsimonious phylogeny, despite its inability to provide unequivocal

support for the human and gorilla grouping (*34*). In both cases, the lack of resolution can be explained according to the three "rules of increasing ambiguity" discussed by W. M. Fitch [*Syst. Zool.* **20**, 406 (1971)] and M. M. Miyamoto [*Copeia* **1986**, 503 (1986)].

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Linkage of Functional and Structural Heterogeneity in Proteins: Dynamic Hole Burning in Carboxymyoglobin

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Inhomogeneous broadening of the 760-nanometer photoproduct band of carboxymyoglobin at cryogenic temperatures has been demonstrated with a dynamic hole burning technique. Line-shape changes and frequency shifts in this spectral band are generated by ligand recombination and are shown not to be the result of structural relaxation below 60 K. The observation of dynamic hole burning exposes the relation between the structural disorder responsible for the inhomogeneous broadening and the well-known distributed ligand rebinding kinetics. The findings provide direct evidence for the functional relevance of conformational substates in myoglobin rebinding. In addition, a general protocol for evaluating the relative contributions of structural relaxation and hole burning to the spectral changes accompanying ligand rebinding in hemeproteins is presented.

A KEY ROLE FOR STRUCTURAL dynamics in understanding protein function is suggested by the kinetics of ligand rebinding in photodissociated carboxymyoglobin (COMb). At room temperature a solution of COMb exhibits bimolecular rebinding kinetics subsequent to photodissociation (*1*). Such simple kinetics indicate that the protein has a single structure or a distribution of structures that fluctuate on a time scale that is fast relative to the rebinding process. For temperatures in the cryogenic regime ($T < 200$ K) the simple kinetic decay becomes a power law, which may be fit by a distribution of exponentials (*2–4*). Distributed kinetics at low temperatures are characteristic not only of hemeproteins but of other protein systems such as the photosynthetic reaction center (*5*). Several explanations for the origin of the power-law kinetics have been offered (*4, 6–9*); however, the ones with the most far-reaching implications for protein properties are based on the concept of conformational substates. In the substate description, every global tertiary structure of a protein has an associated distribution of conformations. These substates, which are separated by potential-energy barriers, may differ only slightly in atomic positions. In this model the different substates have different functional properties. At higher temperatures, thermal fluctuations are sufficient to promote interconversion among the different substates, which gives rise to an averaged structure with simple kinetics, whereas at cryogenic temperatures each molecule is trapped in a specific substate having a distinct kinetic rate constant. The observed kinetics at cryogenic temperatures results from a progressive reaction of a frozen or a slowly relaxing ensemble (*8*). Similar inhomogeneity is expected at higher temperatures for functional events occurring on time scales that are fast compared to the substate interconversion time.

Spectroscopy provides a direct method with which to probe the linkage between functional and structural heterogeneity. For hemeproteins such as hemoglobin and myoglobin there are numerous spectral bands whose intensity reflects the concentration of either liganded or dissociated forms of the protein. Of these spectral bands there are many whose frequencies correlate with structure. Does the spread in frequencies associated with the spectral line shape of a structure-sensitive band reflect the disorder of conformational substates? A spectral band is termed homogeneous if the observed line shape is characteristic of each molecule that contributes to the spectral transition. In this case line broadening can arise from lifetime effects, dephasing, and thermal averaging over protein conformations on a time scale that is fast compared to the characteristic measurement time. If, however, there is either a static distribution of conformations or if the measurement time is fast relative to the substate interconversion time, then each conformational substate contributes its characteristic frequency and line shape to the overall spectral band. If this static or quasi-static structural distribution has a large spread in spectral frequencies relative to other line-broadening mechanisms, then the spectral band is inhomogeneously broadened and different portions of the line shape might then be connected with specific conformational substates.

Several spectral studies have been used to demonstrate structural heterogeneity in proteins, and structural disorder has been directly monitored with x-ray crystallography (*10*); however, spectroscopic techniques for demonstrating structural heterogeneity based on inhomogeneous line broadening are more readily adapted to comparative and time-resolved studies over a large range of solution conditions. These studies typically involve optically accessing a discrete portion of the structural distribution by optically exciting a narrow portion of an inhomogeneously broadened line. This approach, in the guise of photochemical and nonphotochemical hole burning of absorption spectra (*11, 12*) and fluorescence line narrowing of extrinsic (*13*) and intrinsic (*14*) chromophores, has been successfully used to demonstrate inhomogeneous broadening of chromophore-associated spectral bands in proteins. In these techniques selectivity arises from the resonant excitation of those members of the inhomogeneous population having the same optical transition energies; consequently, there is no immediate or necessary connection between disorder probed by these techniques and distributed functional properties detected by other methods, for example, kinetics. In the present work a functional process rather than an optical excitation is used to probe the inhomogeneous properties of a structure-sensitive optical transition, thereby providing the potential for a direct link between conformational and functional heterogeneity.

If an inhomogeneously broadened spectral line characteristic of either the photoproduct or the rebound species has a frequency spread that reflects a distribution of structural parameters controlling the rebinding, then as the ligands rebind the spectral band will not only decrease or increase, respectively, but it will also show frequency changes. For the photoproduct, the early phase of ligand rebinding will remove those frequencies from within the inhomogeneous band corresponding to that part of the population having the fastest rebinding constants. Thus, the rebinding process in effect dynamically burns a hole in the inhomogeneous band in a manner somewhat analogous to the optical hole burning mentioned earlier. Since in the dynamic case those frequen-

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