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

BLAIR F. CAMPBELL, MARK R. CHANCE, JOEL M. FRIEDMAN

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-

AT&T Bell Laboratories, Murray Hill, NJ 07974.

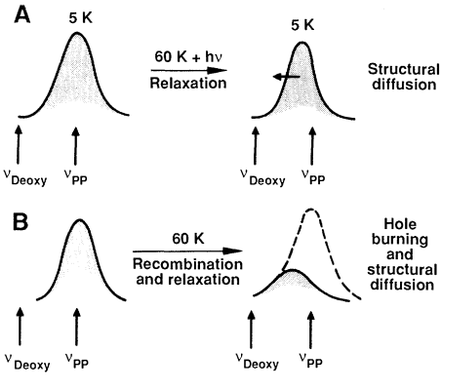

Fig. 1. A schematic representation of protocols (**A**) and (**B**) used in the experiment. The arrows indicate the peak absorption frequency of the deoxy form (ν_{Deoxy}) and the photoproduct (ν_{pp}). The temperature of 60 K is arbitrary in that it is necessary to raise the temperature to a point where a certain extent of rebinding occurs over the duration of time spent at the elevated temperature. Depending on the specific protein, lower temperatures can often be used. In every instance examined (all at temperatures of 60 K or lower), protocol A yields a spectrum that is unchanged from the reference uncycled 5 K spectrum. In all cases where spectral changes occur for protocol B, rephotolyzing also yields that same reference 5 K spectrum.

cies that are lost in the hole burning correspond to fast rebinding structures, a correlation between spectral frequency and specific elements of the protein structure provides an unambiguous connection between parameters of structure and function.

The near-infrared (IR) absorption band at ~760 nm was chosen for the present study because of its many appropriate properties (*15–17*). This band, characteristic of unliganded five-coordinate ferrous hemes, does not appear in the rebound six coordinate spectrum. The peak frequency of this band is responsive to changes in protein structure (*15, 16*) and the line shape appears to be Gaussian even at room temperature, which is suggestive of inhomogeneous broadening (*17*). The charge-transfer character of this near-IR transition (*18, 19*) involves the iron and the surrounding π system of the heme, which may encompass the proximal histidine. Thus, the 760-nm band is a protein structure–sensitive spectral band characteristic of the unliganded binding site, which may be inhomogeneously broadened.

Because of the observed properties of the near-IR band at cryogenic temperatures, a special protocol must be designed to distinguish dynamic hole burning from other sources of spectral change. In the dynamic hole burning experiments, the line shape of the near-IR band is monitored for the photoproduct as a function of ligand recombination. For both myoglobin and hemoglobin, the photoproducts at cryogenic temperatures have their near-IR bands shifted to the red relative to the respective equilibrium deoxy forms (*15, 16*). It follows that relaxation of the nonequilibrium photoproduct spectrum toward the stable deoxy spectrum could easily complicate any observation of frequency or line-shape changes as a function of recombination. Indeed, the time evolution at cryogenic temperatures of the near-IR spectrum in COMb* (the COMb photoproduct) has been interpreted as structural relaxation (*16*).

The protocol shown in Fig. 1 is designed to separate out relaxation from hole burning. A sample of COMb is completely photodissociated at a temperature (~5 K) where there is essentially no recombination. This spectrum of the fully photolyzed sample at 5 K is our reference, reflecting the line shape of the full distribution of photoproduct structures. The sample is then warmed to a temperature where partial recombination occurs. In protocol A the sample remains under illumination at the elevated temperature, whereas for protocol B the sample is maintained in the dark. In both protocols the samples are recooled to 5 K (after being maintained at the higher temperature for some duration Δt) and the resulting near-IR spectra are then compared to the reference spectrum. The absence of light in protocol B allows both recombination and structural relaxation to occur over the interval Δt. In protocol A the sample (by virtue of being illuminated) is both turning over (rephotolyzing and recombining) and being maintained at a higher level of photolysis compared to protocol B. Since the influence of recombination is being reduced while the effective temporal window for structural relaxation is enhanced (by increasing and maintaining the population of photoproduct), the protocol A spectrum should have an exaggerated influence from structural relaxation. The relaxation effects in the protocol A spectrum must be at least as prominent as those occurring for the dark-cycled (same Δt) spectrum of protocol B. On the other hand, dynamic hole burning due to recombination will have much less of an effect on the protocol A spectrum. Consequently, if comparisons are made between the cycled samples and the initial sample, larger changes seen for protocol A are consistent with structural relaxation whereas dynamic hole burning would be indicated by a larger change in the protocol B spectrum.

Figure 2 shows the results of one series of cyclings from 5 to 60 to 5 K for sperm whale COMb* [COMb*(SW)]. Over a Δt of 15 minutes at 60 K approximately 70% of the photodissociated CO recombined in protocol B, whereas very little recombination occurred in protocol A. The protocol A

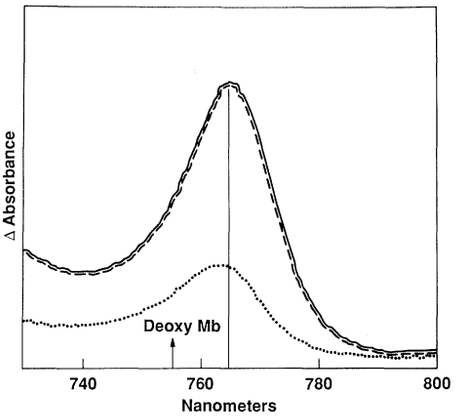

Fig. 2. Absorbance change from carboxymyoglobin: (—), COMb photolyzed at 5 K; (. . .), sample raised to 60 K and allowed to partially recombine for 15 minutes then returned to 5 K (protocol B); (- - - -), sample raised to 60 K and continuously photolyzed for 15 minutes then returned to 5 K with the light on (protocol A). The peak position of deoxymyoglobin is marked by the arrow as a reference. Rephotolyzing the protocol B sample restores the spectrum to one that is identical to the solid line. The photolysis source was an 80-W tungsten projector lamp; the same lamp with 5.0 OD (optical density) attenuation and a Schott OG570 color filter before the sample was used to probe absorbance changes. All spectra were recorded with a fixed setting ISA HR 320 spectrograph (300 grooves per millimeter grating in second order) fitted with a Princeton Instruments OMA detector.

spectrum and the initial fully photolyzed spectrum are virtually identical. The photoproduct spectrum of the surviving 30% in the protocol B spectrum is reduced in intensity and shifted to the blue compared to the initial fully photolyzed spectrum. Similar results are seen for COMb*(horse). The initial spectrum and the normalized protocol B spectrum after ~90% recombination for COMb*(horse) are shown in Fig. 3. In the protocol B spectrum the near-IR band is both shifted to blue and narrower relative to the fully photolyzed spectrum. The spectral narrowing is more pronounced in horse compared to sperm whale. Both the shift and the narrowing increase with increased recombination. In both cases, rephotolyzing the sample at 5 K regenerates the reference spectrum without change in frequency, intensity, or line shape.

The protocol A spectrum is identical to the reference spectrum, whereas the protocol B spectrum is shifted; difference spectra, such as those shown in Fig. 4, enhance our ability to detect changes in frequency and line shape. Since all the difference spectra in Fig. 4 are on the same scale, it is clear that the true protocol A difference (Fig. 4, spectrum B) is negligible compared to the difference shown for the simulated case (Fig. 4, spectrum C). This result, in conjunction

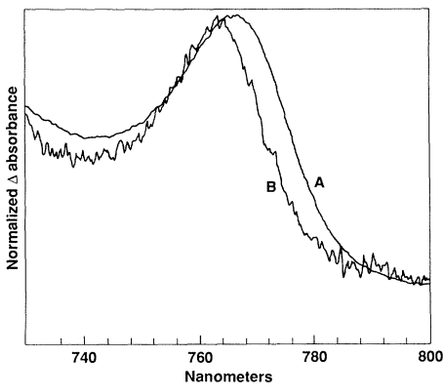

Fig. 3. A comparison of the normalized near-IR absorption (92% recombination) following protocol B (curve B) and the corresponding spectrum of the reference fully photolyzed COMb(horse) sample (curve A). This series of spectra was obtained from a frozen glass (water-glycerol) at neutral *p*H (5 m*M* in myoglobin). Similar results were obtained for COMb(horse) in ice.

with those shown in Figs. 2 and 3, indicates that structural relaxation is not an appreciable factor in the near-IR spectral dynamics of COMb*(SW) at temperatures of 60 K and lower.

The conclusion that the spectral changes indicated in Figs. 2, 3, and 4 are the result of dynamic hole burning and not structural relaxation is supported not only by the preceding arguments but also by the reported absence of an isosbestic point (*16*) and by our preliminary observation that the amplitudes of the protocol B difference spectra scale with the extent of rebinding. These results indicate that the fastest rebinding photoproducts are those that contribute to the low-frequency or red edge of the near-IR band. The occurrence of hole burning clearly demonstrates that the 760-nm band is inhomogeneously broadened. Furthermore, dynamic hole burning dictates that the structural disorder responsible for this inhomogeneous broadening generates the distributed kinetics (*2–4*).

An important next question is the source of the structural disorder responsible for the inhomogeneous broadening. Several studies indicate that protein control of ligand binding occurs at the binding site (*20, 21*). Both distal and proximal heme effects are likely to contribute (*21–23*). Structural heterogeneity in the distal heme pocket is reflected in there being several IR-distinguishable bound CO conformers in myoglobin (*24*). The different CO conformers have different rebinding kinetics and do not interconvert at cryogenic temperatures (*23, 25*). These findings raise the prospect that heterogeneity in the ligand geometry, perhaps as a result of disorder in the architecture of the distal heme pocket, could be the source of the power-law kinetics. However, at cryogenic temperatures there is no hole burning or line-shape change in the individual IR bands (CO stretch) upon ligand rebinding (*23*) and the distributed rebinding kinetics are still observed for individual conformer populations (*23, 25*). These findings suggest that the power-law kinetics originate from a different element of protein structure. We have observed dynamic hole burning in the 760-nm band of the COMb photoproduct from sperm whale and horse at different *p*H values and in both ice and glass (water:glycerol or ethylene glycol). Furthermore, we observe that for horse myoglobin, conditions that substantially change the relative populations of CO conformers (*p*H 4.5 versus 7.5) produce no change in peak frequency or line shape for the 760-nm band. Since these different samples have different populations of CO conformers (as determined by IR), including some with essentially one conformer, the observation of dynamic hole burning in the 760-nm band cannot, to any significant degree, be due to the minimal observed heterogeneity associated with the different CO conformers. But, the data do not prove the existence of a continuous distribution of conformational substates, only a number of states.

Protein-induced variations in the proximal heme pocket appear to play a role in the control of ligand binding properties (*20–22*). A correlation between the yield of geminate recombination and the iron proximal-histidine stretching frequency seen in the resonance Raman spectra of various hemoglobin photoproducts led to the suggestion that the activation energy barrier for ligand binding is modulated by the protein through the proximal-histidine (F8) iron linkage (*21, 22*). A distribution of histidine-heme geometries would imply a distribution of activation energy barriers; therefore, if the Raman band were inhomogeneously broadened, it might reflect a mapping of the energy barrier distribution onto the spectral distribution, although there is no prior reason for assuming this. Attempts at dynamic hole burning of the iron-histidine stretching mode in the picosecond time-resolved (~50- psec resolution) Raman spectrum of O_2Hb* at ~4°C by means of the geminate recombination of O_2 were unsuccessful (*26, 27*). Given the recent dynamics simulation (*28*) showing substate interconversion in myoglobin on the 2- to 10- psec time scale, it is possible that on the time scale for the hole burning experiment (tens of picoseconds), a given protein molecule samples the full distributions of conformational substates; that is, the population is homogeneous on the tens of picoseconds time scale. Raman hole burning experiments at cryogenic temperatures have proved to be exceedingly difficult. However, there are indications that the frequency shifts in the iron-histidine mode are reflected in frequency changes in the 760-nm band. For example, in the comparisons of Mb(SW) versus COMb*(SW) and HbA versus COHbA*, the Raman iron-histidine band is shifted to higher frequency while the 760-nm band is shifted to lower frequency. Furthermore, the room temperature relaxation kinetics for the 760-nm band and the iron-histidine Raman band are quite similar in the photoproducts of COHb and COMb (*17*). If the disorder in the iron-histidine mode is responsible for the distributed kinetics, then the hole burning pattern observed in myoglobin implies that the fastest rebinding conformational substates are those with the highest frequency iron-histidine. This population would, on the basis of the structural assignment for the variations in the iron-histidine frequency, correspond to the least tilted or strained geometries of the heme-histidine unit (*29, 30*).

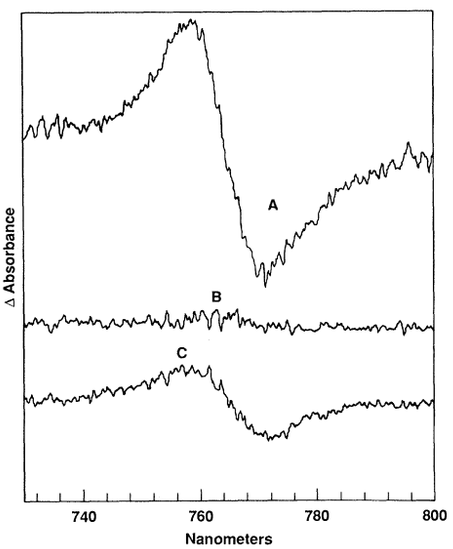

Fig. 4. Difference spectra derived from the absorption spectra shown in Fig. 2. Spectrum A is the difference between the normalized spectrum at 5 K derived from protocol B (15 minutes at 60 K without light) and the initial photoproduct spectrum at 5 K. Spectrum B is the difference between the protocol A spectrum at 5 K (15 minutes with illumination) and the reference 5 K spectrum of the photoproduct. The difference spectrum (C) is generated by taking the difference between a spectrum composed of the reference 5 K photoproduct spectrum (multiplied by 2/3) and the unnormalized protocol B spectrum (in which 1/3 of the sample remains photodissociated) and the reference photoproduct spectrum. Spectrum C is what would be expected for spectrum B if spectrum A originated from structural relaxation and the 1/3 unrecombined population in this protocol B were the only part of the distribution to undergo relaxation. In other words, spectrum C is the minimum difference expected for the protocol A minus reference difference (spectrum B) if structural relaxation is responsible for the protocol B shifts in frequency and/or line shape.

In conclusion, it has been shown that the 760-nm absorption band of the cryogenically trapped photoproduct of COMb is inhomogeneously broadened with respect to ligand rebinding subsequent to photodissociation. Hole burning occurs in this absorption band as the fastest rebinding members of the total photoproduct population undergo recombination. This result provides a direct link between the power-law kinetics and the distributed structural element that maps onto the inhomogeneous line shape of the band. It is possible that the distributed structural element that gives rise both to the spread in frequencies in the 760-nm band and to the distribution of barrier heights responsible for the power-law kinetics is associated with the heme–proximal-histidine linkage. The dynamic hole burning technique provides a direct method of probing at elevated temperatures fast, functionally linked equilibrium fluctuations that result in the interconversion of conformational substates.

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