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IS IT ONLY RELAXATION?
INHOMOGENEOUS KINETICS IN PROTEINS AND SOLUTION

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ABSTRACT
The transient shift in the near IR absorption spectrum of myoglobin at cryogenic temperatures and in the fluorescence spectrum of an aromatic dye molecule (coumarin 153) have been previously interpreted as (protein, solvent) relaxation. A similar shift may result from a combination of inhomogeneous line-broadening and inhomogeneous kinetics, now known as "kinetic hole burning". We summarize methods for distinguishing between inhomogeneous and relaxational mechanisms. These methods show that the low temperature shift in the near IR band of myoglobin is due only to inhomogeneities, while the (high temperature) dynamic Stokes shift in coumarin 153 may be inhomogeneous at short times and relaxational at long times.

"Pardon me," said Milo to the first man who happened by,
"can you tell me where I am?"
"To be sure, you're on the Island of Conclusions. Make yourself at home.
You're apt to be here for some time."
"But how did we get here?" asked Milo, who was still a bit puzzled by being there at all.
"You jumped, of course. That's the way most everyone gets here".
Norton Juster, "The Phantom Tollbooth".

1. Introduction
Both processes of ligand binding to heme proteins\textsuperscript{1-12} and transient Stokes shifts in solvated dye molecules\textsuperscript{11-20} involve transitions between two states: The unbound and bound states for heme proteins; the ground and electronic excited states for the dye molecules. These states are presented schematically in Figure 1 as a function of a protein/solvent coordinate. The transitions between the two states are ligand rebinding following photodissociation of bound heme proteins and (radiational and non-radiative) decay of the excited (singlet) state following laser excitation of ground-state dye molecules. In both cases one follows these transitions spectroscopically in the time-domain: By transient absorption\textsuperscript{6-8} in the case of heme proteins and time-resolved fluorescence\textsuperscript{4-10} for the excited dye molecules. Finally, in both cases one observes transient spectral shifts which were attributed to protein\textsuperscript{1} or solvent\textsuperscript{18-19} relaxation.
Figure 1. A unified schematic representation of ligand binding and solvation.

The question raised in this presentation is whether a transient shift necessarily represents relaxation? One alternate interpretation, which does not involve relaxation at all, may be an inhomogeneous ("kinetic hole-burning") mechanism. This mechanism assumes that both spectral and kinetic properties depend on the value of a protein/solvent coordinate. As a result, fast reacting conformations disappear first from the spectrum which therefore shifts towards wavelengths characterizing the slower reacting conformations. In Section 2, a simple test is presented that may help distinguish between these two mechanisms. In Sections 3 and 4 this test is applied to low-temperature CO binding to myoglobin and to the transient Stokes shift of coumarin 153 in propanol. These examples show how important kinetic information may be obtained from the observed shifts.

2. Inhomogeneous Kinetics vs Relaxation

In order to demonstrate the essentials of the two mechanisms, let us consider the simplest possible model in which two conformations, A and B, are characterized by different wavelengths $\lambda$ in the (absorption or emission) spectrum. Assume the peak of conformation A is centered at $\lambda = 1/4$ (in some arbitrary units) and has a homogeneous width of 1/2. The peak for B is centered at $\lambda = 3/4$ and has the same homogeneous width of 1/2. Hence the total linewidth is 1 and the "observed" average wavelength is 1/2, as seen in Figure 2. The assumed kinetics will now lead to variations in both average wavelength and total area as a function of time.

Figure 2. A schematic representation of the initial lineshape.
In a relaxational mechanism we may assume that \( A \to B \) with a rate coefficient \( k \). If initially \( [A] = [B] = 1/2 \), the time dependence of the concentrations in this simple scheme will be

\[
[A(t)] = \frac{1}{2} \exp(-kt), \quad [B(t)] = 1 - \frac{1}{2} \exp(-kt)
\]

In this mechanism, therefore, the total area is constant, \( a(t) \equiv [A(t)] + [B(t)] = 1 \), while the average wavelength shifts from its initial value, \( 1/2 \), to a final value of \( 3/4 \) according to

\[
\bar{\lambda}(t) = \frac{1}{4} [A(t)] + \frac{3}{4} [B(t)] = \frac{1}{4} [3 - \exp(-kt)]
\]

The point to note here is that area and shift are completely uncorrelated: Variation in \( k \) affects the shift but not the area.

In the simplest inhomogeneous ("kinetic hole burning") mechanism, conformation \( A \) decays with a rate coefficient \( k \) (due to a reaction such as ligand binding or decay to the ground state of an electronically excited state) while state \( B \) is inert. Hence \( A \to \text{Products} \) in first-order kinetics while \( [B(t)] = [B(0)] = 1/2 \), so that the total area diminishes as

\[
a(t) = \frac{1}{4} \left[ 1 + \exp(-kt) \right]
\]

The average wavelength is again obtained by weighing the peaks of the individual lines, \( 1/4 \) and \( 3/4 \), by the normalized population in the two states

\[
\bar{\lambda}(t) = \frac{1}{4} [A(t)] + \frac{3}{4} [B(t)] = \frac{3 + \exp(-kt)}{4 \left[ 1 + \exp(-kt) \right]}
\]

One may again check that \( \bar{\lambda}(0) = 1/2 \) while \( \bar{\lambda}(\infty) = 3/4 \).

The interesting observation is that, unlike in the relaxation mechanism, for the inhomogeneous mechanism both area and shift depend on \( k \), which may be eliminated between Eqs. (3) and (4) to yield a "universal" relation

\[
\bar{\lambda}(t) = \frac{1}{4} \left[ 1 + a(t)^{-1} \right]
\]

of peak area and frequency. The correlation in Eq. (5) is independent of both time and the numerical value of the rate coefficient. One may vary this rate coefficient by orders of magnitude for example, by changing the temperature, and still expect the data of shift vs area to fall on the same "universal" curve. While the exact functional form of such a curve is model dependent, the existence of the correlation is much more general. It is like cutting off pieces from a nice Bavarian cake: As the mass of the cake diminishes its center of gravity shifts. The relation between the two depends only on the size of the slice, but not on the rate at which it has been sliced away.

Figure 3. The slicing of a Bavarian cake.
Therefore when a kinetic hole-burning mechanism is operative, a temperature-independent correlation between the wavelength of maximal intensity and the area of the spectral lineshape is predicted.19 In addition, if the spectral line is symmetric, one may expect transient line-narrowing.19 If, on the other hand, relaxation is the dominant mechanism, there is no reason to expect either one of the above effects. In the following, we will apply the above criterion to test experimental data of low-temperature CO binding to myoglobin and the so-called "solvation dynamics" of coumarin.

3. CO Binding to Myoglobin

Myoglobin (Mb) is a globular protein whose main function is to store oxygen in the muscle. It is a "simplified" version of hemoglobin, as it is built of a single polypeptide subunit containing (in sperm whale Mb) some 153 amino acids, folded into 8 a-helix segments. The prosthetic group which is responsible for Mb function is a porphyrin ring, which complexes at its center a ferrous (Fe2+) iron cation. Four of the ligands in the octahedral coordination of the iron are nitrogen atoms in the porphyrin ring, the fifth is a nitrogen atom in the imidazole ring of the amino acid histidine (His F8, the 8th residue on the F-helix, also known as the "proximal histidine"). Through the proximal histidine the porphyrin is attached to the protein backbone. The sixth coordination site is used to bind a ligand (O2, CO). The vacancy in the protein matrix on the distal side where the ligand binds is known as the "heme pocket".

![Heme pocket](image)

Figure 4. The structure of myoglobin. Left is the tertiary structure of the protein (globin) with its 8 helices denoted by the letters A-H and showing the location of the heme group and heme pocket. Right is the heme group (an iron porphyrin) which comprises the active site of heme proteins.

When Mb is bound to a ligand (six-coordinated iron) the porphyrin structure is planar while in the unbound ("deoxy") state it is domed, the iron being 0.2-0.5A out of plane in the proximal direction. The spin states of bound and deoxy Mb also differ. The bound state is a singlet (S=0) while the deoxy state is a quintet (S=2). As a result of these differences in structure and spin states, there are many differences in the absorption spectra shown in Figure 5. The most prominent feature in these spectra is the intense peak between 400 and 450nm, known as the "Soret" band and attributed to a π→π* transition of the porphyrin ring. The peak upon which attention is focused below is the weak feature near 760nm observed for deoxy-hemes (the "near IR" or band III) and assigned to a porphyrin to iron electron transfer (σπ→σμ transition).
The mechanism is operative, a wavelength of maximal intensity in addition, if the spectral narrowing is on the other side no reason to expect either apply the above criterion to test myoglobin and the so-called main function is to store hemoglobin, as it is built of a single Mb with 153 amino acids, which is responsible for Mb its center a ferrous (+2) iron nation of the iron are nitrogen atom in the imidazole ring of the F-helix, also known as the line the porphyrin is attached e is used to bind a ligand (O₂), tal side where the ligand binds.

The tertiary structure of the letters A-H and showing Right is the heme group of heme proteins.

Ordinated iron the porphyrin \( \pi \) state it is domed, the iron ion. The spin states of bound singlet \( S=0 \) while the deoxy differences in structure and spin in spectra shown in Figure 3. Intense peak between 400 and d to a \( \pi \rightarrow \pi^* \) transition of the is focused below is the weak e near IR e band III) and \( \pi_n \rightarrow \delta_{sz} \) transition).

Figure 5. The absorption spectra of carbon monoxide (full curve) and deoxy myoglobin (dotted curve) at 20K, showing both Soret and near-IR bands. Adapted from Ref. 3.

The differences in the absorption spectra enable one to follow the rebinding kinetics of a ligand (CO, for example) to deoxy-Mb subsequent to a short photolyzing laser pulse applied to the bound sample (MbCO, for example). Following the photolyzing pulse the Fe-CO bond cleaves and the iron moves into the domed, \( S=2 \), position on a sub-picosecond time scale. At later times rebinding begins. Below the glass transition of the solvent (ca. 200K for typical solvents) the ligand (CO) cannot escape to solution. It is trapped in the heme pocket from which it eventually rebinds. The cryogenic kinetics of CO rebinding to Mb (as a function of temperature and over many decades in time) was pioneered by the Urbana group. Typical data, obtained in the Soret regime and shown in Figure 6a, are strongly non-exponential in time. This has been interpreted as evidence for a distribution of protein conformations, which are trapped at cryogenic temperatures without the ability to interconvert. One might imagine the effect of these conformations as modulating the iron out-of-plane distance around its average domed structure: Conformations for which the iron is closer to planarity bind faster since they resemble the geometry of the bound state. Above the glass transition, when conformations can relax, the kinetics gradually (with increasing time and temperature) becomes exponential, as seen in Figure 6b.

Figure 6. Kinetics of CO binding to myoglobin. Plotted is the survival probability of deligated Mb following a short laser pulse (a) at cryogenic temperatures and (b) above the glass transition. Note the log-log scale.
The origin of the distribution of barrier heights can be understood with the aid of the two dimensional potential energy surface. Figure 7, suggested by Agmon and Hopfield, suggests the iron-CO distance, so that the well on the right is the heme pocket. The vertical axis is a "protein coordinate," along which the protein, perhaps, to the iron displacement between the two spin states. In the kinetic coordinate at the glass transition temperature. Photolysis carries this population horizontally over the potential surface, thus preparing a non-equilibrium distribution of protein conformations of deoxy-Mb. At cryogenic temperatures, this non-equilibrium distribution is unable to relax, so that rebinding is a horizontal (Frank-Condon like) process, resulting in different barriers for different protein conformations. Above the glass transition, protein conformations may relax, leading to a transition from non-exponential to exponential kinetics. The shift between the minima along the protein coordinate is a necessary requirement for fitting the low temperature data. If this shift is indeed coupled to proximal iron displacements, then the model predicts that conformations with the iron closer to planarity react faster. Hence both relaxation and rebinding tend to move the iron further away from planarity.

![Figure 7. A Mb-CO potential surface showing rebinding paths for varying relaxation rates.](image)

It has been postulated that protein distributions that affect the kinetics are real and not just a mental construction for explaining experiment. This would be the case if both reaction rates and spectral transitions are inhomogeneously distributed and the absorption frequency is a simple monotonic function of the protein coordinate. Under such conditions spectral lines will shift due to rebinding at low temperatures, and due to both rebinding and protein relaxation at high temperatures. This is seen in Figure 8. When the relaxation amplitude is large compared with the homogeneous width the spectral line can "step out" of its initial envelope. For a while, no real effort was devoted to the detection of such a mapping between protein conformations and spectral frequencies.
In 1985 the Urbana group has published an investigation of spectral shifts observed in the near-IR band of Mb during low temperature ligand rebinding. As can be seen from Figure 9, this peak decays with time and shifts slightly to the blue, even at temperatures as low as 40K. The amplitude (or area) decay of the near-IR band matches that which was previously observed in the Soret (Fig. 6a) and was therefore attributed to ligand rebinding. More puzzling was the origin of the spectral shift, which has suggested to the researchers a relaxation process in some protein mode.

How can there be relaxation at such low temperatures where kinetic studies have established the existence of frozen protein substates? This has been rationalized with the aid of a postulated "ultrametric" ("fractal") structure for protein conformational substates, as depicted in Figure 10. These states are organized in levels (tiers), each state on a given level is split into many substates with smaller barriers, comprising a lower lying tier with a similar but scaled structure. According to this picture, it is the first level of protein conformations which is frozen while the lower tiers may still relax. As the temperature is lowered, additional tiers become frozen.
Figure 10. The assumed ultrametric structure of protein conformations.5
Every well in the upper tier (dashed curve) contains a scaled down structure of secondary wells (full curve). According to Ref. 5, relaxation could occur in the lower tier (arrows).

Considering the above relaxational explanation for the observed shift in the near-IR band, one concludes that conformations on the lower tier must induce inhomogeneous line broadening on that level, otherwise relaxation could not be observed spectroscopically. If this is so, why not expect even larger inhomogeneous broadening for the higher tiers? But the first tier shows also kinetic inhomogeneities. The combination of both kinetic and spectral inhomogeneities must lead to a kinetic hole-burning mechanism at least in addition to (if not instead of) relaxation.

To test whether the mechanism is relaxational or inhomogeneous, the original data5 has been subjected to the test described in Sec. 2 namely, the spectral shift was plotted as a function of rebinding (peak’s area). As can be seen from Figure 11a, a universal plot was obtained in the temperature range 60-160K, but only after an assumption was made concerning the zero-time origin of the peak.10 Such an assumption was necessary because microsecond excitation is not sufficiently fast for the higher temperatures studied. The experiments were therefore repeated at Urbana1 with improved accuracy over the temperature range 25-75K. The results are reproduced in Figure 11b. Indeed a beautiful universal correlation is observed, which strongly suggests an inhomogeneous mechanism.

Figure 11. Shift vs area of the near IR band adapted from Refs. (a) 10 and (b) 5.
Transient line narrowing is more difficult to detect due to the large homogeneous width of the peak (ca. 20nm FWHM) compared with shifts on the order of 1nm. At the higher temperatures studied, the variation of the width with time seemed to be statistical noise. However, for the 60K data, systematic line-narrowing has been detected as can be seen in Figure 12 below. This effect is expected for an inhomogeneous mechanism but generally not for relaxation.

![Figure 12](image)

Figure 12. Transient line narrowing detected at the lowest temperature reported in Ref. 4, after data smoothing with a SVD routine.

The first experiments demonstrating that the low temperature shifts involve zero relaxation were the temperature cycling experiments conducted at Bell Labs by Friedman and collaborators. MbCO was photolized at 5K, where no rebinding occurs, and its near IR spectrum recorded. It was then heated to 60K under intense illumination where it was kept for 15 min. Under the conditions that light intensity is sufficiently strong to keep the sample photolized throughout the heating cycle, relaxation at the higher temperature is encouraged. Once the sample is cooled back to 5K and rephotolized, it should show a blue shift if any relaxation has occurred at 60K. The experiment showed no shift whatsoever, hence all of the observed shift at 60K is due to kinetic hole-burning. A similar T-cycling experiment for horse Mb (Figure 13) clearly demonstrates the kinetic hole burning effect as missing intensity in the red wing of the spectrum. These experiments have not yet been repeated at higher temperatures where, due to faster rebinding, even stronger illumination sources are required.

![Figure 13](image)

Figure 13. Kinetic hole burning in the near IR band of horse Mb. Adapted from Ref. 8.

adapted from Refs. (a) 10 and (b) 5.
It remains to consider why the fast binding conformations are red shifted in the near IR band. This can be understood with the aid of a crystal-field correlation diagram, as shown in Figure 14. The electronic configuration of the ferrous ion in the high-field hexacoordinated bound state is the low-spin $t_{2g}^6e_g^2$, with a total spin zero. As the 6th ligand is removed on the distal side, the ligand field decreases and one observes the small-field high-spin electronic configuration $d_{x^2-y^2}, d_{xy}, d_{z^2}, d_{xz}, d_{yz}$, resulting in a total spin of 2 for the deoxy state. As seen from Figure 14, the degeneracy of the $t_{2g}$ states has been removed: Those orbitals with a z-component have gained extra stability from ligand dissociation. Hence $d_z$ is lower in energy than $d_x$, so that the lowest electronic transition from the porphyrin π system is to $d_y$. The latter has some z-character, so it is destabilized by out-of-plane motion of the iron which increases its interaction with the proximal histidine. As a result we expect the π→$d_y$ transition to blue-shift the further the iron is displaced from the porphyrin plane. But by our earlier assumption, the larger the proximal displacement the slower the rebinding rate. Hence one concludes that blue conformations are slow binding whereas the red conformations are fast binding and therefore disappear first from the spectrum.

![Diagram](image)

Figure 14. Crystal field correlation diagram for the iron $d^6$ configuration:
From a strong octahedral field, through a weak octahedral field to level splitting due to symmetry reduction induced by removal of the ligand.

Finally it is noted that inhomogeneous broadening has been observed in other bands of the Mb absorption spectrum shown in Figure 5. In optical hole burning experiments inhomogeneous broadening of the wide Soret band has been detected. It has been argued that the inhomogeneous broadening of the Soret correlates to a much smaller degree with the distributed kinetics. This is expected from the electronic nature of the electronic transition involved: A π→π* transition should indeed be less sensitive to iron location than a σ→d transition. If the influence of protein conformation on the kinetics is through modulations in out-of-plane iron displacement, the major spectroscopic effect would be on transitions involving iron orbitals.

A spectral shift following rebinding was recently detected also in the IR frequency of the CO stretching mode (the $A_1$ state). Since there are not yet complete data of shifts and amplitudes, definite conclusions from this observation may be premature. It is hoped that future work will yield complete time-resolved IR spectra which will be amenable to analysis such as shown in Figure 11.
Informations are red shifted in the aid of a crystal-field electronic configuration of the state is the low-spin $t_{2g}$, on the distal side, the ligand $d_{z^2}$ electronic configuration for the deoxy state. As seen been removed: Those orbitals n ligand dissociation. Hence electronic transition from the some $z$-character, so it increases its interaction with $\tau-d_x$ transition to blue-shift $\pi$ plane. But by our earlier he slower the rebinding rate, slow binding whereas the red at first from the spectrum.

$$\begin{align*}
  &\rightarrow \frac{1}{d_{x^2-y^2}} \\
  &\rightarrow \frac{1}{d_{y^2}} \\
  &\rightarrow \frac{1}{d_{x^2}} \\
  &\rightarrow \frac{1}{d_{xy}} \\
  &\rightarrow \frac{1}{d_{xz}} d_{yz} \\

the iron $d^6$ configuration: k octahedral field to level normal of the ligand.
\end{align*}$$

4. Dynamic Stokes Shifts

When an aromatic dye molecule is excited to its first singlet state, the change in electronic distribution often leads to an increase in dipole moment. If the molecule is solvated in a polar solvent, the favorable orientation of solvent dipoles differs between the ground and excited states. As a result, the potential curves are shifted along the solvent coordinate as seen in Figure 1. Consequently, the fluorescence maximum is red-shifted relatively to the absorption peak. This red shift has been observed since many years in steady-state (constant illumination) spectra.11–13

Nanosecond spectroscopy has first revealed this solvatochromic shift in the time domain.14 With the development of pico and femtosecond lasers it became possible to follow the transient shift in many dye molecules even at room temperature.15–18 In the simplest case of a dye molecule which cannot undergo a chemical reaction (electron transfer or isomerization19) spectral shifts reflect the stabilization of the excited state by the solvent. In the literature14–17 it has been taken for granted that the solvent effect is dynamic namely, solvent dipoles relax and recrystallize around the newly created excited-state dipole. It is, however, not inconceivable that a solvent behaves like glass for very short times and relaxes on longer time scales. It is therefore natural to ask whether all of the observed shift represents relaxation or else some part of it is due to static inhomogeneities?

To apply the suggested test of plotting shift vs area, a good temperature series is needed for a simple dye molecule that (hopefully) does not undergo any complicating chemical reactions. It has been suggested12 that such a molecule is coumarin 153, whose structure is shown in Figure 15. Because the amino group is tied to the aromatic ring it cannot undergo internal twisting, known to be coupled in such molecules to intramolecular charge transfer.21 Since Moncecol and Fleming29 have recorded transient fluorescence spectra of coumarin 153 in n-propanol over the temperature range 222–298K, this molecule is a reasonable candidate for testing22–23 the proposed12–14 relaxational mechanism.

![Figure 15. The structure of coumarin 153.](image)

As the mechanism was (and still is) widely believed to be solvent relaxation, the information in the spectral amplitudes was considered uninteresting, so the reported fluorescence data were all normalized to a common height.22 To apply the present analysis the information in the amplitudes is indispensable. The transient spectra were therefore reconstructed.26 The original data, in the form of transient decay curves at different wavelengths, were reassembled to give transient spectra at varying time delays (Figure 16). The relative normalization at different wavelengths was determined from the measured steady-state spectrum which should equal to the time integral of the transient spectra. As seen from Figure 16, the shift at room temperature is very fast and therefore the spectrum at long times has the same shape as the steady-state spectrum (plus signs). At low temperatures the shift is slow and consequently the steady-state spectrum is blue-shifted compared with the long-time spectrum.
Figure 16. Transient fluorescence spectra of coumarin 153. Top to bottom: 20, 50, 200, 500, 2000 and 5000 ps after the excitation pulse. Plus signs denote the steady-state spectrum (normalized to the intensity at 5 nsec).

When frequency shift and area (or intensity) are drawn separately as a function of time one obtains the results shown in Figure 17. Neither shift or area decay exponentially, though their deviation from exponentiality is not as large as in the myoglobin data since the time dependence can adequately be represented by a bi-exponential. The transient behavior of both area and shift is strongly temperature dependent, the higher the temperature the faster the change. We observe a systematic trend with temperature, excluding the room temperature data (not shown) where most of the shift occurs within the instrument response function (110 ps).

Figure 17. Frequency shift (left panel) and area decay (right panel) of the transient fluorescence spectra of coumarin 153 in n-propanol at (top to bottom) 222, 233, 253 and 273K.
When the data in the two panels of Figure 17 is unified in one plot, Figure 18, the different decay curves at the four temperatures merge and one obtains a unified curve at short times (when the area is large), which seems to split at longer times. This behavior suggests that approximately half of the shift is due to inhomogeneities. At later times, there is a crossover from inhomogeneous kinetics to relaxation occurring, roughly, 100-200 psec after excitation at 273K and 500-1000 psec at 222K. While these times may seem long for solution phase kinetics, it should be noted that the Debye relaxation times in n-propanol in these temperatures are 1-10 nsec and even the longitudinal relaxation times (140-1200 psec) are quite long in comparison.²²

![Graph showing frequency vs area decay of coumarin 153 fluorescence in n-propanol at temperatures 273K, 253K, 233K, and 222K.](image)

Figure 18. Frequency shift vs area decay of coumarin 153 fluorescence in n-propanol in the temperature range 222-273K. Note that data points in the upper right corner correspond to short times whereas points in the lower left corner represent long times.

If indeed an inhomogeneous mechanism is operative in this system it requires that the rate coefficients differ by orders of magnitude between the blue and red edges of the spectrum. It is known¹⁹ that the radiative rate for these dye molecules is larger in the blue, but the variation over the relevant spectral region is typically by a factor of 2.3. It is plausible, therefore, that an inhomogeneous effect is due to an additional radiationless decay mechanism which is considerably faster for solvent conformations typical to a ground state probe molecule. Indeed it has been determined¹⁹ that while the absolute quantum yield of coumarin 153 in hydrocarbon solutions is close to unity, it drops in alcohols to a value below 0.4. (This is quite in contrast to the analogous coumarin derivative whose CF3 group is replaced by a methyl group, which shows a solvent insensitive quantum yield.) The nature of this radiationless mechanism, the major decay channel of excited coumarin 153 in propanol, is yet to be established.

Finally, spectro-kinetic inhomogeneities could be detected by several other ways. At cryogenic temperatures, where solvent relaxation ceases, one should still see shifts due to the kinetic hole-burning mechanism. Indeed such shifts have recently been found²¹ in glasses at 80K. This suggests performing a temperature cycling experiment.²² In the limit that the spectral width of the exciting laser is infinitely narrow, inhomogeneities are removed and all the observed shift will be due to relaxation. Differences in the kinetics of samples excited at the red and blue edges of their absorption band are yet another indicator of inhomogeneous effects.
5. Conclusion

The present representation has dealt with sample inhomogeneities which effect both spectral widths and reaction rates. When conformations that absorb or emit at one edge of the spectrum differ considerably in their reaction rates from conformations on the other spectral edge, transient shifts will be observed. These shifts may be (and indeed have been) confused with conformational relaxation. To differentiate between inhomogeneous kinetics (kinetic hole-burning) and relaxation, a simple test has been devised for plotting spectral shifts against areas. It is suggested that a universal, temperature independent correlation is a clear fingerprint of an inhomogeneous mechanism.

The above test has been applied to data from both CO binding to myoglobin and Stokes shifts in a coumarin dye. For the myoglobin case the suggested test together with other experimental evidence has altered the interpretation of the low temperature shift in the near IR band, now believed to be due only to the kinetic hole burning mechanism. This inhomogeneous explanation necessarily means that protein (or solvent) conformations are simply mapped to spectral frequencies. Hence by performing multiple (rather than single) wavelength experiments one may actually follow the time evolution of the conformational distribution responsible for the inhomogeneous kinetics. This observation opens the road to a new generation of kinetic experiments on heme proteins.

In the case of the transient Stokes shift in coumarin the evidence at present is much less conclusive. If spectro-kinetic inhomogeneities do play an important role in this system as well, theories for explaining "spectral relaxation" may be irrelevant to experiment. It is therefore imperative to verify the origin of the spectral shifts before elaborate theories for explaining solvent relaxation are developed. As always, science will reach conclusions by traversing the bumpy road of collecting new experimental evidence.

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