Spurious conformational transitions in proteins?
(protein dynamics/stochastic processes/D-amino acid oxidase/fluorescence)

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ABSTRACT Temperature-dependent dynamic processes in biological macromolecules can produce sharp and reversible transitions in spectroscopic properties that might be misinterpreted as evidence for thermally induced conformational changes. This provides a rational explanation for the paradoxical case of D-amino acid oxidase [D-amino-acid:oxygen oxidoreductase (deaminating), EC 1.4.3.3], for which a sharp fluorescence transition at 14°C, not observed by sensitive calorimetry [Sturtevant, J. M. & Mateo, P. L. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 2584-2587], could be due to a dynamic quenching process of large activation energy, rather than a change in conformational state of the protein. Similar interpretations may be valid in other systems studied by experimental techniques that depend, directly or indirectly, on molecular relaxation processes.

Abrupt transitions in spectroscopic properties of proteins are conventionally ascribed to conformational change. This may be misleading, because spectral properties can be affected by other processes not necessarily associated with discernible changes in polypeptide geometry. In the case of D-amino acid oxidase [D-amino-acid:oxygen oxidoreductase (deaminating), EC 1.4.3.3] Massay et al. (1981) found a 30% decrease in tryptophan fluorescence intensity upon raising the temperature of the protein solution from 8°C to 20°C. The transition was sharp and reversible, with a midpoint at about 14°C, and was interpreted as a thermally induced isomerization between two distinct conformational states of the protein separated by an enthalpy difference, \( \Delta H \), of about 78 kcal mol\(^{-1}\) (1 kcal = 4.18 kJ). Interpretation of the more ambiguous discontinuities in absorbance and kinetic properties of the enzyme in the same temperature region (1) was complicated by subunit and cofactor dissociation equilibria. Subsequent work by Sturtevant and Mateo (2) confirmed the fluorescence transition, though with a lower apparent \( \Delta H \) of about 50 kcal mol\(^{-1}\) for the flavoprotein, and 32 kcal mol\(^{-1}\) for the apoenzyme. Their calorimetric studies, however, failed to show any comcomitant change in heat capacity or enthalpy of the protein under identical conditions. This unequivocally rules out any thermodynamic transition and suggests that the fluorescence intensity reflects something other than the average conformational state of the protein (2).

Fluorescence is a stochastic phenomenon. It depends not only on intrinsic properties of the fluorophore but also on dynamic rate processes involving transient events such as molecular collisions, reorientations, and energy transfer during the lifetime of the fluorescent excited state (3-7). Various functional groups within proteins are capable of quenching tryptophan fluorescence, including amines, carboxylic acids, sulphydryls, and imidazole groups. Additional quenching can arise from energy transfer between suitably oriented aromatic groups and by interaction with solvent and solute molecules (3-7). All these can be dynamic processes requiring only transient interaction with the excited group. They may, therefore, be temperature dependent.

The fluorescence quantum yield, \( F \), of a species A may be written:

\[
F = \frac{k_l}{k_l + k_m},
\]

in which \( k_l \) and \( k_m \) are the rate constants for the radiative and nonradiative decay processes (\( h \) is the Planck constant):

\[
A^* \rightarrow A + h\nu \quad \text{and} \quad A^* \rightarrow A + \text{heat}.
\]

Nonradiative decay may occur by a variety of mechanisms. Intrinsic processes, including intersystem crossing and internal conversion, will depend on the chemical nature of the molecule and bulk properties of the environment (polarity, dielectric constant, etc.) and will be relatively insensitive to changes in temperature per se, though they might be affected indirectly by conformational changes in a protein that modify the surrounding of the fluorescing groups. On the other hand, rates of dynamic quenching involving molecular motions and activation processes will vary with temperature. \( k_m \) may therefore be written as the sum of temperature-dependent and temperature-independent terms:

\[
k_m = k_s + \frac{k_B T}{h} \sum k_i \exp(-\Delta G_i^T/RT),
\]

in which the temperature dependence has been written in the form of an absolute rate theory expression with activation free energies \( \Delta G_i^T \) for each of the available mechanisms. \( k_B \) and \( R \) are the Boltzmann and gas constants, respectively, and \( T \) is the absolute temperature.

Taking a simplified form, in which only one temperature-dependent process dominates, one obtains an expression for the fluorescence quantum yield that varies with temperature:

\[
F(T) = \frac{k_l}{k_l + k_s + B T \exp(-\Delta H^T/RT)},
\]

in which

\[
B = \frac{k_B}{h} \exp(\Delta S^T/R)
\]

and the activation enthalpy and entropy contributions to \( \Delta G^T \) have been separated.

There is nothing new here. The thermal quenching of fluorescence of aromatic compounds in aqueous solution has been described, with activation energies for indole derivatives as high as 12 kcal mol\(^{-1}\) (4). What is significant, in the present context, is that this expression is a sigmoidal function of temperature and, for large activation energies, can give rise to abrupt changes in fluorescence emission over a narrow temperature range.
range. Furthermore, this temperature dependence is numerically indistinguishable from the variation one would expect from a two-state equilibrium transition.

Given a fluorescence change, and assuming it to be a result of an equilibrium change in conformation, one might obtain the equilibrium constant for the transition from:

\[ K = \frac{F_o - F(T)}{F(T) - F_w} \]

in which \( F_o \) and \( F_w \) are the low- and high-temperature limits of the fluorescence, respectively. The equilibrium constant would be related to the thermodynamic parameters for the transition in the usual way:

\[ K = \exp(-\Delta G/RT) = \exp(\Delta S/R)\exp(-\Delta H/RT), \]

and a linear van’t Hoff plot of \( \ln(K) \) vs. \( 1/T \) would yield estimates for equilibrium enthalpy and entropy changes.

If, on the other hand, the fluorescence transition were due to the thermal quenching process of Eq. 1, then substitution in Eq. 2, together with the limiting values

\[ F_o = \frac{k_i}{k_i + k_o} \]

would give an apparent equilibrium constant of the form

\[ K_{app} = \frac{BT}{k_i + k_o} \exp(-\Delta H^2/RT). \]

Apart from the relatively slowly varying preexponential term, \( K_{app} \) shows the same functional temperature dependence as \( K \) and, over moderate temperature ranges, would give an apparently linear van’t Hoff plot despite the absence of any equilibrium transition. The slope of such a graph gives the activation enthalpy \( \Delta H^2 \) for the dynamic quenching process and not the change in heat content of the system.

This is borne out numerically for d-amino acid oxidase in Fig. 1, in which the experimental data of Massey et al. (1) are compared to a theoretical curve of the form of Eq. 1, with an activation enthalpy of 78 kcal mol\(^{-1}\) and activation entropy of about 254 cal K\(^{-1}\) mol\(^{-1}\), assuming a typical value of the order of 1 nsec for the fluorescence lifetime \((k_i + k_o)^{-1}\). It is clear that a dynamic quenching mechanism describes the data equally as well as a two-state conformational transition, and in view of the calorimetric evidence (2) must be considered a likely alternative for d-amino acid oxidase.

The physical picture might be as follows. Proteins are dynamic objects and, in common with all matter, consist of atoms and groups in perpetual motion at a thermal equilibrium (8–10). At low temperatures such motions might be relatively small and slow, so that fluorescence quenching is dominated by static properties of the system—\( k_0 \) of Eq. 1. At higher temperatures, however, the rates of dynamic processes in the protein will increase exponentially. Some of these transient molecular motions may be capable of quenching the excited state of one, or more, of the tryptophan residues in the protein at rates that will rapidly overtake \( k_0 \) to give a sharp decrease in fluorescence emission. It must be clearly stated that no conformational change is implied here. The average coordinates of the atoms and groups in the macromolecular do not change but, rather, the frequency and, possibly, the amplitudes of motion about these mean positions increase.

To explain the d-amino acid oxidase phenomenon one must envisage motions in the protein at rates of \( 10^8 \) sec\(^{-1}\), or more, at 14°C and with activation energies of order 78 kcal mol\(^{-1}\). At first sight these may seem rather large numbers, but standard thermodynamic fluctuation theory (8) shows that for d-amino acid oxidase with a monomer molecular weight of about 45,000, the root-mean-square deviation in equilibrium energy is about 50 kcal mol\(^{-1}\). For a normal statistical distribution this implies that of order 5% of the molecules at thermal equilibrium have internal energies in excess of 78 kcal mol\(^{-1}\) above the mean at any one time (i.e., every d-amino acid oxidase molecule spends 5% of its time above this energy). Molecular mechanics simulation (9) shows that protein atomic movements in excess of 1 Å may occur on a picosecond time scale, and rotational motion of tryptophan residues at rates up to \( 10^{10} \) sec\(^{-1}\) have been observed in some proteins (11). On the slower time scale available to NMR, aromatic ring rotations with activation enthalpies up to 37 kcal mol\(^{-1}\) can be detected (12). Fluorescence quenching by these and other sorts of motion is clearly feasible, and high activation parameters could result if the quenching mechanism required the simultaneous transient fluctuation of several groups. [Compare, for example, the kinetics of cooperative thermal unfolding transitions of globular proteins (13–15), in which activation energies and entropies of order 70 kcal mol\(^{-1}\) and 200 cal K\(^{-1}\) mol\(^{-1}\), or higher, have been observed.]

D-Amino acid oxidase may be an extreme example, but there is no reason to believe that it is unique, or that fluorescence intensity is the only property that might be affected by dynamic processes. Indeed, any observable property whose magnitude depends on stochastic relaxation processes is potentially open to discrete changes whenever two, or more, relaxation mechanisms with different thermal coefficients are present. This might include such additional properties as fluorescence depolarization and lifetimes, NMR and ESR characteristics, enzyme kinetics, chemical reactivity, and so on. The dangers inherent in the solely structural interpretation of discontinuities in enzyme kinetics have been pointed out by Sturtevant (2) and by Londesborough (16). It now appears that even the apparently more direct probes of protein conformation will have to be examined more circumspectly, and similar problems may afflict techniques that purport to measure lipid phase transitions in biological membranes.
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