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KINETICS OF REVERSIBLE PROTEIN DENATURATION. A STUDY ON APLYSIA MYOGLOBIN

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The kinetics of denaturation and renaturation of Aplysia Myoglobin by temperature-jump and stopped-flow are reported. The time course of the unfolding and refolding reveals the presence of significant amounts of intermediates both in absence and in the presence of alcohols. A linear three states reaction mechanism is proposed and discussed.

1. Introduction

Aplysia Myoglobin undergoes a completely reversible denaturation whose equilibrium properties have been previously described [1] in the framework of a simple two-states denaturation model.

In this paper we deal with the kinetic aspects of the same process and discuss the data with reference to a two-states kinetic model.

Dynamic studies of protein unfolding under condition of true reversibility have been carried out on different systems, mostly simple globular proteins, but also on conjugated proteins such as cytochrome c [2–4]. While the equilibrium picture generally conforms to a simple two-states model, the kinetics of unfolding shows frequently more than one kinetic process, both by rapid-mixing and (or) relaxation methods. The observation of more than one step in the pathway of denaturation implies the presence of significant amounts of at least one intermediate, in contradiction to the equilibrium formulation. Some of the more complete sets of data recently obtained have been discussed in an attempt to characterize the intermediate and its properties with reference to either the native or the denatured forms. One of the hypothesis recently advanced will be considered in the discussion, and the kinetic data on Aplysia Mb, reported below, will be discussed with reference to this hypothesis. It may be advanced that the kinetics of unfolding of Aplysia Mb is not consistent with a two-states kinetic model, contrary to the indications obtained from the equilibrium investigation.

2. Materials and methods

Aplysia myoglobin was prepared and purified as previously reported [5,6].

Temperature jump experiments were performed with a single beam apparatus manufactured by Messanlagen (Göttingen, Germany). A temperature-jump cell of 7 ml capacity and 1 cm light path was used; changes in absorbancy in the Soret region at suitable wavelengths were used to follow the kinetics of conformational changes. Temperature jumps in the range 0.5–5°C were imposed by suitable discharge of the condenser.

Rapid mixing experiments were carried out at constant temperature with a Durrum stopped-flow apparatus, equipped with a 2 cm observation cell.

All the kinetic experiments were performed in 2% borate buffer pH 9.15. Ethanol or n-butanol reagent grade from Merck (Darmstadt) were used as chemical denaturants.

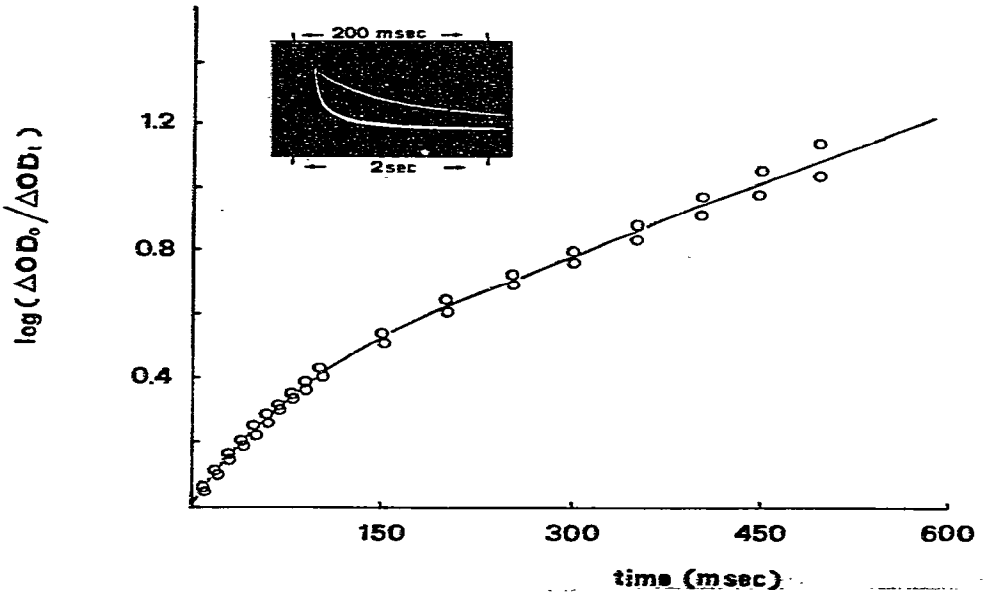


Fig. 1. Time course of a temperature-jump experiment on Aplysia Mb in 2% borated buffer pH 9.1 containing 4% (v/v) n-butanol. Initial temperature 40.5°C corresponding to a value of $\theta = 0.3$. 8 kV discharge corresponding to $\Delta T = 0.4$°C. ΔOD_0 (0.069) represents the absorbance change between time zero and infinity (base line). ΔOD_t the absorbance change from time t and infinity. The solid line represents a fit of the experimental points to the equation $\log(OD_0/OD_t) = \log(A_1 + A_2)/[A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)]$, where $A_1 = 0.037$; $A_2 = 0.032$; $\tau_1 = 5.1 \times 10^{-2}$ s, $\tau_2 = 2.9 \times 10^{-1}$ s. The insert reports the CRT record of the experiment; vertical scale 0.01 volt/cm; horizontal scale 20 and 200 ms/cm (upper and lower scales respectively).

3. Results

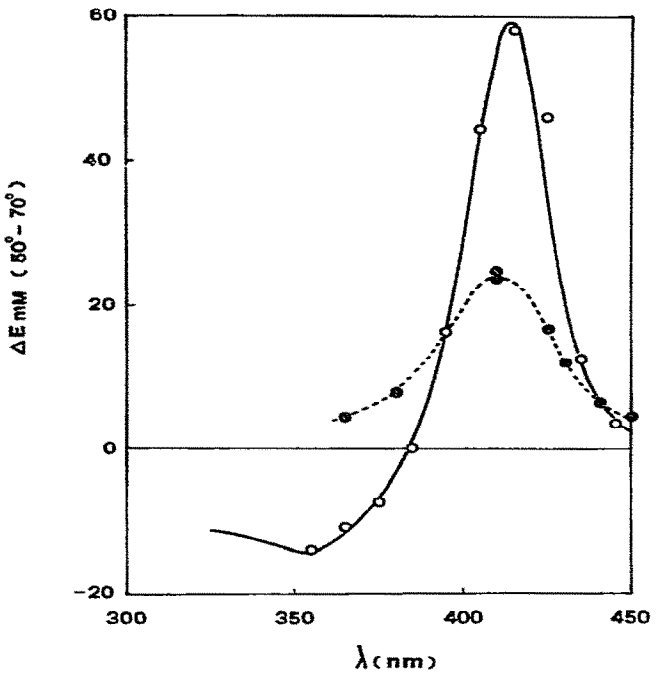
3.1. Temperature-jump experiments

Temperature-jump experiments were performed on solutions of Aplysia Mb in 2% borate buffer (pH 9.15) in the absence or in presence of alcohols (ethanol or n-butanol) at various concentrations.

Appropriate temperature jumps were induced to give final temperatures in the unfolding range. The resulting relaxation event, observed through changes in absorbance at wavelengths from 350 to 450 nm is shown, under one experimental condition, in fig. 1, from which it can be seen (a) that the reaction occurs in the millisecond time range and (b) that the relaxation process cannot be described in terms of a single exponential.

The assignment of the observed relaxation to a perturbation of the equilibrium between the native and denatured forms of the protein has been made on the basis of the following observations: i) the total change in absorbancy depends on the observation wavelength, following a difference spectrum which corresponds to that obtained statically between the native and denatured protein (fig. 2); ii) the amplitude of the process, in any given solvent, depends on the final equilibrium temperature showing a maximum corresponding to the mid point of the transition at equilibrium, and decreasing towards zero amplitude outside the transition range (fig. 3).

It must be noticed that besides the main, well resolved process, a rapid, non-resolvable, phase is observed. Considering the dead-time of our temperature-jump instrument under the conditions used, this unresolved phase must have a relaxation time smaller than 5 μs. The unresolved phase is not related to a perturbation of the ionization equilibrium which involves deprotonation of the water molecule coordinated with the heme iron at the sixth position [7]. This conclusion is based on the observations that (i) the wavelength dependence of the amplitude is different from that involved in the ionization of the



water molecule; (ii) at the pH of the experiments, the only species significantly populated is the alkaline form ($Fe^{+3} - OH^-$), since the pK of this transition is 7.4 at 40°C; (iii) the relaxation process for the ionization of the water molecule was easily resolved by temperature jump ($\tau^{-1} \approx 165\ s^{-1}$ at pH 9.1 and 40°C) [7].

Moreover we have evidence that this unresolved phase is not directly related to the main unfolding transition, as shown by the findings that (i) its amplitude is independent of θ and (ii) its wavelength dependence is different from that characteristic of

Fig. 2. Difference spectrum between native and denaturated Aplysia Mb is shown as a continuous line. Open circles indicate the kinetic difference spectrum, obtained by temperature-jump under the same conditions (i.e. 2% borate pH 9.1) normalized to the static spectrum. The dotted line and solid circles indicate the kinetic difference spectrum of the initial unresolved phase observed in temperature-jump experiments, plotted in arbitrary units. It is important to realize that in the middel range of the transition this phase contributes less than 5% to the total amplitude.

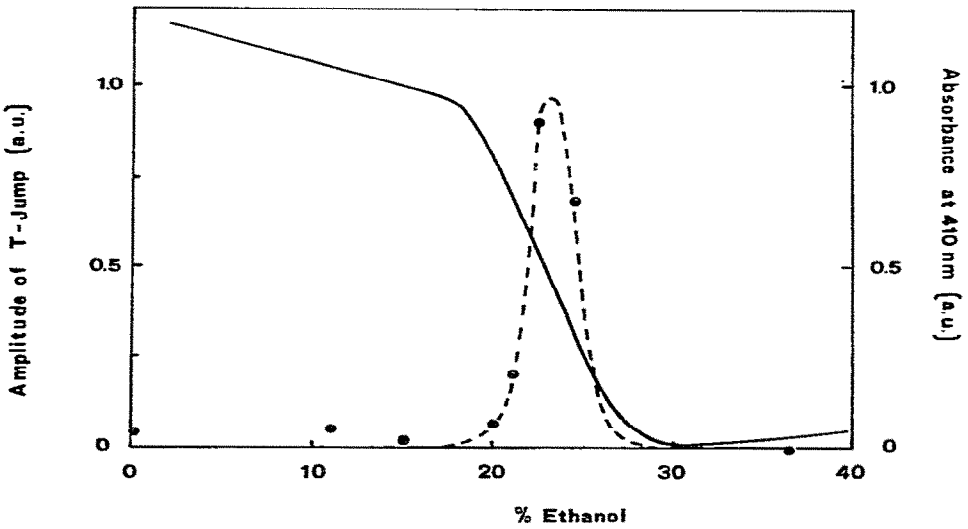


Fig. 3. Dependence on ethanol of the absorbance at 410 nm of a solution of Aplysia Mb, at the constant temperature of 36°C, is indicated as a continuous line. The total amplitude observed in temperature jump experiments performed in parallel is indicated as a function of the percent of ethanol with closed circles and dotted line. The change in temperature was 1.5°C in all cases to get the same final temperature, observations at 410 nm.

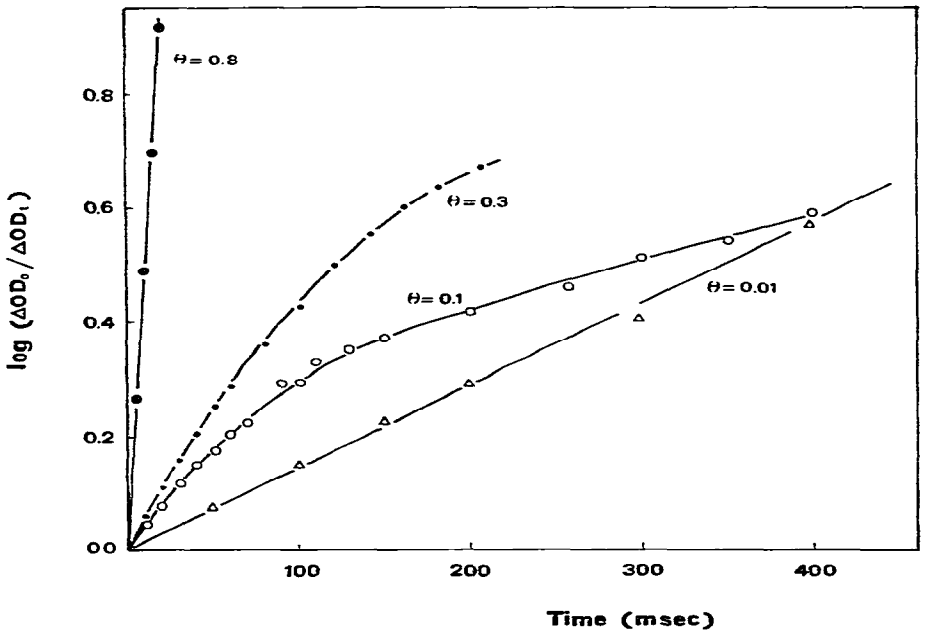


Fig. 4. Time course of the relaxation kinetics as observed for a solution of Aplysia Mb starting at different values of θ (indicated) obtained by progressive increase of n-butanol concentration from 1.96 to 4.9% (v/v). Starting temperature 41°C; temperature jump = 1.4°C; observation wavelength = 412 nm.

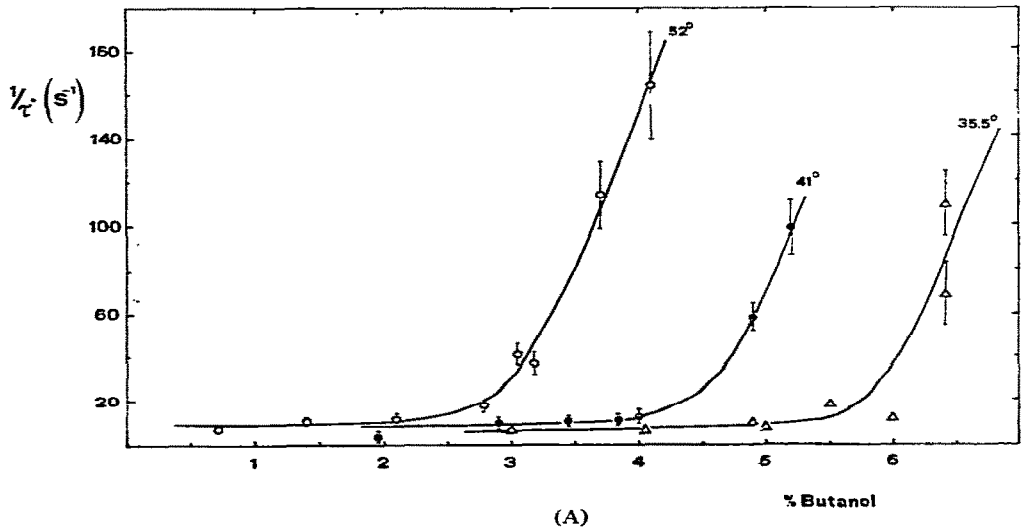
the denaturation process (fig. 2). The interpretation of this very fast, unresolved, event is at the moment unclear, although it may be possible to correlate this observation with the temperature-dependent spin state changes according to the information now available for hemoglobin [8].

The analysis of the relaxation behaviour of the system under various conditions gives rise to the following results:

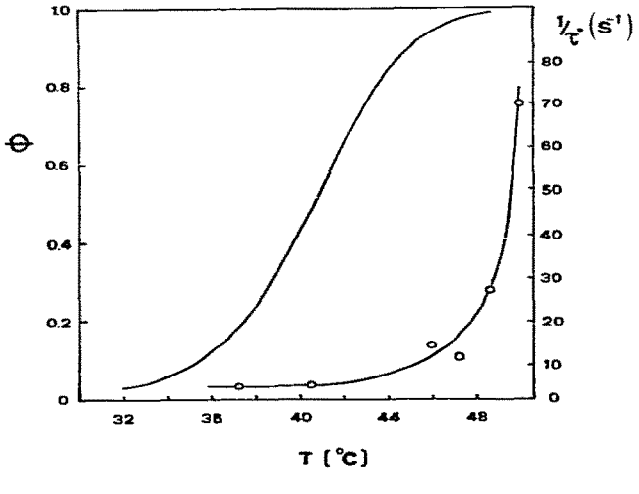
(1) The shape of the relaxation process depends on the extent of transition at equilibrium, quantitatively defined by θ ‡. Over most of the transition region the relaxation time course cannot be analysed in terms of a single relaxation time, and at least two exponentials, must be used to fit the data (fig. 1). In the middle range the presence of more than one relaxation time course is independent of the size of the temperature change (from 0.5° to 5°C) i.e. of the extent of the perturbation $\Delta\theta$ from ~ 0.05 to ~ 0.3). The analysis of the relaxation spectrum provides, under some conditions a satisfactory fit of the relaxation time course with two exponentials (see fig. 1). However this conclusion cannot be extended, with reasonable confidence, to the whole range of conditions explored. Thus given the errors in the signal and base line determination, we prefer to analyse and present the results in terms of the mean reciprocal relaxation time, as defined by Schwartz [9].

(2) At low or high values of θ, i.e. close to the end points of the unfolding transition, the relaxation appears to correspond to a single process (fig. 4). A dependence on θ of the relaxation time course and the value of the mean reciprocal relaxation time is observed both at constant solvent composition, if the temperature is varied over the unfolding range and at constant temperature, if the solvent composition is varied (fig. 5A and B). In both cases $(\tau^*)^{-1}$ increases abruptly towards the end of the transition.

‡ The overall extent of denaturation θ was calculated in parallel equilibrium experiments from the static optical density changes at one wavelength as follow: $\theta(T, C) = [(A_N - A)/(A_N - A_D)]_{T,C}$, where A_N and A_D are the absorbancies of the native and denatured forms at temperature T and solvent composition C.



(A)



(B)

Fig. 5. (A) Dependence of $1/\tau^*$ on the per cent (v/v) of n-butanol at three different final temperatures (indicated). Discharge of 16 kV, corresponding to a temperature-jump of 1.4°C. Mb concentration 10 μM. (B) Dependence of $1/\tau^*$ on the final temperature for a solution of Aplysia Mb (10 μM) containing 18% (v/v) of ethanol. The thermal transition under identical conditions is shown as a continuous line. The final temperature was reached using different temperature-jump between 0.5 and 5.0°C.

(3) The shape, rate and wavelength dependence of the process are independent of protein concentration (from 2×10^{-6} to 2×10^{-5} M).

3.2. Stopped-flow experiments

Similar experiments were performed by the rapid-mixing method, imposing a change in solvent composition at constant temperature. This method allows either to increase or to decrease the fraction of denaturated material, and eventually to impose much wider perturbations than it may be possible by the temperature-jump technique.

Insofar as the solvent perturbation is kept relatively small, the results obtained by flow were identical to those described above, independently of the direction of the perturbation. The time course of renaturation, shown in fig. 6, indicates a trend which resembles that given in fig. 4.

The kinetic pattern appears different when large solvent composition changes, which cover a larger fraction of the transition, are involved. In this case the time course of the process depends on the direction of the perturbation, being essentially monophasic in the direction of denaturation, but definitely biphasic in the opposite direction (fig. 7).

The kinetic difference spectrum calculated at various times after mixing, presented in fig. 8, shows that over the range covered only two spectral species are involved, since a neat isosbestic point is observable.

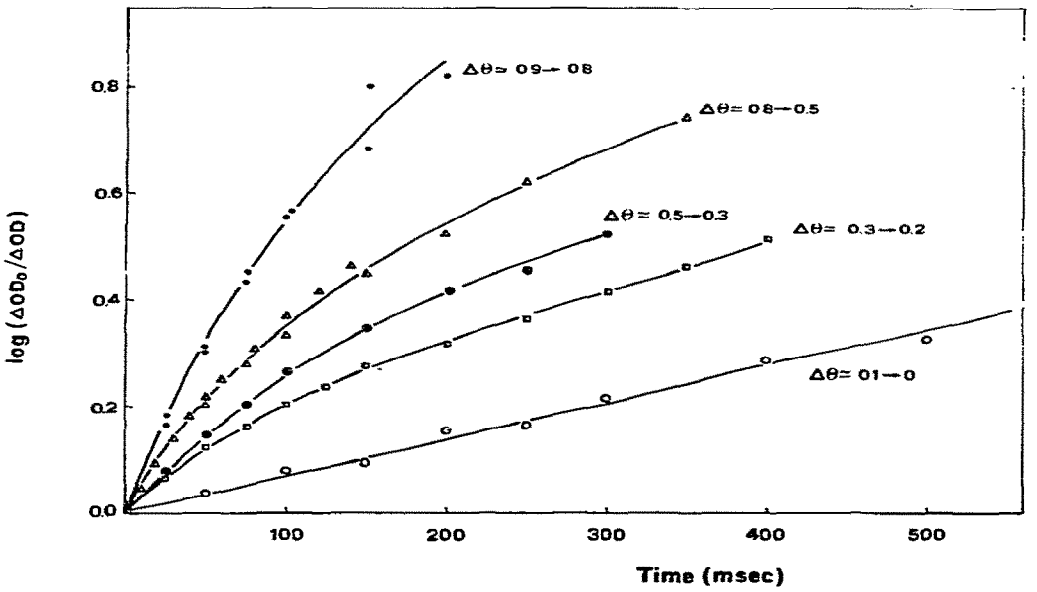


Fig. 6. Time course of renaturation as observed in stopped-flow experiments at constant temperature (= 40°C) imposing small solvent perturbation, corresponding to progressive dilutions of butanol. The change in θ observed in each experiment is indicated in the figure. Mb concentration = 10 μM; observation wavelength = 412 nm. A similar set of results (not shown) was obtained for denaturation.

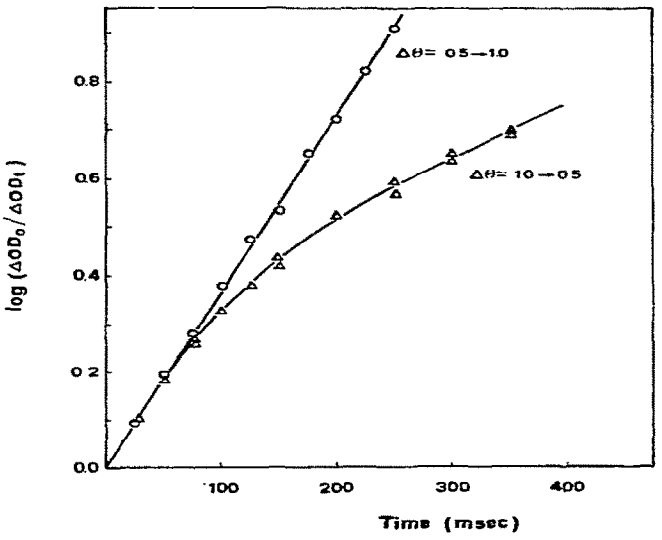


Fig. 7. Time course of renaturation (triangles) and denaturation (circles), as observed in stopped-flow experiments at constant temperature (36°C) imposing large solvent perturbations. The observed changes in θ, indicated in the figure, were obtained by suitable changes of the butanol concentration. Other experimental conditions as in fig. 6.

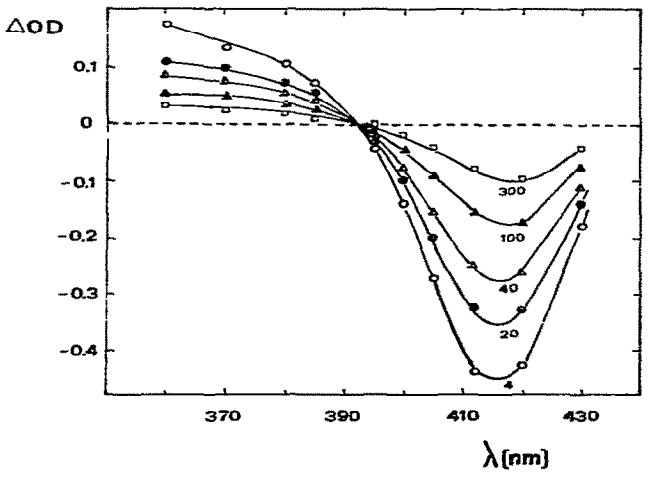


Fig. 8. Kinetic difference spectra observed in a stopped-flow experiment at different times (indicated in milliseconds after mixing) during renaturation. Conditions: Mb at a concentration of ~ 10 μM; in borate buffer containing 30% (v/v) ethanol mixed with buffer, at 35°C. The dilution of ethanol induces a jump from $\theta \approx 1.0$ to $\theta \approx 0.05$.

4. Discussion

The equilibrium picture of the unfolding of a number of proteins has been generally described within a two-states approximation, taking into account thermodynamic information based on calorimetric and spectroscopic measurements [10–12]. On the other hand, as briefly mentioned in the introduction, kinetic investigations have often revealed heterogeneous behaviour. Without attempting to summarize the extensive findings of the literature, it may be sufficient to recall a few of the more recent investigations on this aspect of the problem.

Temperature-jump and stopped-flow studies of the unfolding of ribonuclease have revealed the existence of a fast kinetic process (in the millisecond time range) in addition to a much slower event which occurs in the second time range [2,13]. On the basis of extensive results, a linear three-states model with two unfolded species characterized by different refolding rates has been proposed [14–16]. The model, which also accounts for some of the equilibrium results, implies that the two unfolded forms have indistinguishable physical properties (with the exception of a small pK difference [17]), and the ratio of their population at equilibrium is not significantly temperature dependent. The features of this model have been chosen to be consistent with the characteristic result that the equilibrium transition is independent of the physical property employed to monitor unfolding.

A structural interpretation of such an intermediate stage in the reaction scheme has been advanced by Brandts and coworkers, who suggested that the slow step in protein unfolding is due to a cis-trans isomerization of one or more proline residues [18]. The same feature is suggested to apply also to other proteins, such as chymotripsinogen and cytochrome c. Thus according to this view-point the slow event observed in several cases is not intimately connected to the unfolding process itself, but is related to a change between two conformers of the totally unfolded species. Such a feature would allow to maintain a two-states model description as a general feature of protein unfolding. However, as discussed below, the results on the denaturation kinetics of Aplysia Mb indicate that this view, although valid in some cases, is not always adequate.

It is important to emphasize once again that the heterogeneous time couse of the kinetics of denaturation of Aplysia Mb is in no way related either to protein heterogeneity, since it is well established that the preparation yields a single protein component, or to the size of the perturbation, as shown by the results of both temperature-jump and flow experiments.

It has been shown that native Aplysia myoglobin contains a high degree of secondary structure, and in fact, according to preliminary X-ray crystallographic information [19] and theoretical calculations [20], its tridimensional structure is similar to that of sperm whale myoglobin.

The conformational change involved in the denaturation process does not lead to an unfolded state; although large optical and fluorescence changes, as well as functional modifications, are associated to denaturation, the transition leads to a molecule which still maintains a considerable amount of secondary structure [1]. The absorption features of the denatured myoglobin indicate that heme is still bound to the protein, and by analogy with other denatured hemeproteins, is presumably bound as an hemichrome in the heme pocket. In addition, the lack of effects of protein concentration on the relaxation kinetics indicates that a bimolecular process is not involved. These arguments, as well as other indirect considerations (e.g. free heme is known to polymerize), lead us to conclude that the denaturation is not associated with liberation of heme free in solution.

Although it is obvious that some of the interactions of the heme with the residues in the heme-pocket must be modified on denaturation, we have no direct information on other region(s) of the molecule which may be involved in the conformational change. Thus we cannot exclude that one or more of the six proline residues in Aplysia myoglobin may be affected by denaturation.

Two of the findings reported above are relevant at this point, namely: (a) the spectral properties of the kinetic intermediate(s) are in between those of the native and denatured states, as shown for example in fig. 8; (b) the slower relaxation observed in the middle range of the transition is considerably faster than the slow phase in refolding reported for a number of other monomeric globular proteins. For example, while the slow relaxation determined for the experiment of fig. 1 is 3.4 s^{-1} at 40°C, the slow

step in the denaturation is: 0.1 s^{-1} for chymotripsinogen and 0.5 s^{-1} for cytochrome c (as calculated at 40°C from the reported activation energies) [18].

As outlined above, these slow-steps observed for several proteins have been interpreted as due to a cis-trans isomerism about proline peptide bonds in the unfolded state.

In view of the arguments discussed here we conclude that the slow proline isomerization, although certainly valid to interprete rate limiting steps prior refolding in other proteins, does not provide a convincing interpretation for the biphasic kinetics of denaturation of Aplysia myoglobin. Thus the kinetic intermediate(s) identified in the latter case has to be considered a true intermediate in the pathway of denaturation.

Therefore, along the lines suggested by Baldwin and associates [16], a sequential mechanism, involving at least one intermediate state seems more appropriate to describe the kinetics of Aplysia denaturation:

$$N \rightleftharpoons (X)_n \rightleftharpoons D. \qquad (1)$$

In relation with this mechanism, an important experimental result is represented by the dependence on θ of both the mean reciprocal relaxation time, $(1/\tau^*)$, and the shape of the reaction time course. The same general behaviour is observed in temperature-jump and stopped-flow experiments irrespective of the method of perturbation. The dependence of reaction velocity on θ shows an abrupt increase towards fairly high values of θ (see for example fig. 5), which is also qualitatively consistent with the features of scheme [1]. In fact, changes in solvent composition and/or temperature alter the equilibrium population of the three conformational states which the protein can reversibly assume during denaturation. If we give the meaning of a formal reaction coordinate to the parameter θ, than: a) for values of θ approaching zero the first equilibrium will dominate, and therefore, in agreement with the experimental data, a slow and essentially monophasic relaxation will be observed; b) on the other hand, for values of θ approaching one, the D conformation will be preferentially populated, in agreement with the observation of a monophasic and rapid relaxation; c) for values of θ ranging between these extremes, both processes contribute to the observed relaxation, which will be a function of the relative populations and rate constants involved in the scheme.

The stopped flow experiments for large perturbations also seem in agreement with scheme (1). As shown in fig. 7, a different time course is observed for denaturation and renaturation, although the range of the transition covered in the two experiments is the same (i.e. θ 0.5 ⇌ 1.0). The observation of monophasic time course in the direction of denaturation and biphasic for renaturation suggests the presence of a rate limiting step in the last stages of renaturation.

Scheme (1) is somewhat analogous to the sequential model for the nucleation dependent protein folding proposed by Tsong and coworkers for ribonuclease [21], with the difference that in the case of Aplysia Mb the rate limiting step of renaturation is the attainment of the final native structure rather than the formation of the first nucleus of structure, as in the case of ribonuclease. This result is not surprising for Aplysia Mb, in view of the facts that the denatured state is not totally unfolded and that the heme may play the role of a nucleation center in the denatured form.

In conclusion, the kinetics of denaturation of Aplysia Mb indicates that this process involves at least three conformational states. At present it is not possible to assess the structural differences among the states, especially in view of the limited information still available on the denatured state and on the alleged intermediate(s). However we would conclude from our analysis that the two-states model, although generally useful and undoubtedly valid in many cases, is not applicable to every protein denaturation process. This may be especially the case when considering proteins containing a bulky prosthetic group.

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