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Thermostability of Irreversible Unfolding α-Amylases Analyzed by Unfolding Kinetics*

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For most multidomain proteins the thermal unfolding transitions are accompanied by an irreversible step, often related to aggregation at elevated temperatures. As a consequence the analysis of thermostabilities in terms of equilibrium thermodynamics is not applicable, at least not if the irreversible process is fast with respect the structural unfolding transition. In a comparative study we investigated aggregation effects and unfolding kinetics for five homologous α-amylases, all from mesophilic sources but with rather different thermostabilities. The results indicate that for all enzymes the irreversible process is fast and the precedent unfolding transition is the rate-limiting step. In this case the kinetic barrier toward unfolding, as measured by unfolding rates as function of temperature, is the key feature in thermostability. The investigated enzymes exhibit activation energies (E_a) between 208 and 364 kJmol^{-1} and pronounced differences in the corresponding unfolding rates. The most thermostable α-amylase from *Bacillus licheniformis* (apparent transition temperature, $T_{1/2} \sim 100$ °C) shows an unfolding rate which is four orders of magnitude smaller as compared with the α-amylase from pig pancreas ($T_{1/2} \sim 65$ °C). Even with respect to two other α-amylases from *Bacillus* species ($T_{1/2} \sim 86$ °C) the difference in unfolding rates is still two orders of magnitude.

Due to substantially different environmental conditions of habitats where the residentiary organisms have to thrive, proteins can be provided with very different thermostabilities. In the past two decades numerous proteins from extremophilic organisms have been isolated and characterized with respect to their thermal stability (1–4). A considerable number of proteins has been identified, which maintain their folded (and in general functional) state at rather elevated temperatures, such as 100 °C and above. Interestingly, extreme thermostabilities are not confined to proteins from thermophiles and hyperthermophiles but can also be found for proteins from mesophiles. In particular *Bacillus* species are able to populate moderately thermophilic habitats (5), which at least partly explains the occurrence of extreme thermostable proteins in *Bacillus* strains. An example we will discuss in more detail here is given by the starch-degrading enzyme α-amylase. Several rather heat-stable α-amylases were isolated from mesophilic sources (see TABLE ONE).

Thermal stability of proteins includes thermodynamic as well as kinetic stability. Thermodynamic stability, in the most general case, is determined by the difference in the free energy ΔG_{unf}[2] between the folded (N) and the unfolded state ($\Delta G_{unf} = \Delta H - T\Delta S$). An illustrative parameter given by the melting temperature T_m (as obtained at $\Delta G = 0$) is quite often used to compare thermostabilities of individual proteins. Unfortunately, the most mesophilic and thermophilic proteins unfold irreversible, which in general excludes a quantitative determination of thermodynamic parameter. Only in very rare cases and under specific assumptions were thermodynamic parameter obtained from non-reversible unfolding transitions (6–8). According to the transition state theory where the folded and unfolded states are separated by an energy barrier, kinetic stability is determined by the activation energy of unfolding $\Delta G^{\#}_N$ (see for example Ref. 9). This kind of stability is often characterized by its half-life times ($t_{1/2}$) at defined temperatures.

As a typical representative for a medium-sized multidomain protein (~60 kDa) α-amylases in nearly all cases unfold irreversible (10–13). The only known exception is a psychrophilic α-amylase from *Alteromonas haloplanctis* that exhibits a remarkable degree of reversibility (~99% recovery of the calorimetric enthalpy change, ΔH_{cal}) for such a large protein structure (14). Besides some kinetic studies on thermoinactivation of BLA and BAA (10–12, 15, 16), most comparative studies on the thermostability of homologous α-amylases have been analyzed in terms of melting temperatures (13, 14, 17–21). In some cases additional thermodynamic parameter such as ΔC_P and ΔH were determined (13, 14, 22). In the case of irreversible transitions, the obtained values, which rely on equilibrium thermodynamics, can be under kinetic control and might be therefore strongly biased by heating rates (6, 23). To obtain more reliable and meaningful results aiming to compare thermostabilities of different irreversible unfolding proteins, we focus here on measuring unfolding kinetics. For this purpose a simple kinetic model based on the Lumry-Eyring (24) equation is employed to compare the unfolding kinetics of five different α-amylases.

EXPERIMENTAL PROCEDURES

Enzymes—α-Amylase from *Bacillus licheniformis* (BLA, purchased from Sigma), from *Bacillus amyloliquefaciens* (BAA, from Fluka), from *Bacillus subtilis* (BSUA, from Fluka), and from *Aspergillus oryzae* (TAKA, from Sigma) was obtained as lyophilized powder. A further α-amylase from pig pancreas (PPA, from Roche Applied Science) was obtained in ammonium sulfate solution. Powders were dissolved in buffer (for details see below), and all enzymes were purified by the use of a desalting column (Econo-Pac 10 DG, Bio-Rad). For spectroscopic studies the following buffers were used: 30 mM Mops, 50 mM NaCl, 2 mM $CaCl_2$, pH 7.4 (standard buffer), 30 mM Mops, 50 mM NaCl, 5 mM EDTA, pH 7.4 (calcium free samples). In the case of CD-spectroscopy respective buffers were used, but with 10 mM Mops.

Fluorescence Spectroscopy—Intrinsic fluorescence was measured using a RF-1501 Fluorospectrometer (Shimadzu). Excitation wave-

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[2] The abbreviations used are: ΔG_{unf}, Gibbs free energy change; BLA, *B. licheniformis* α-amylase; BAA, *B. amyloliquefaciens* α-amylase; BSUA, *B. subtilis* α-amylase, TAKA, α-amylase from *Aspergillus orzae*; PPA, α-amylase from pig pancreas; CD, circular dichroism; $T_{1/2}$, apparent transition temperature; ΔH, enthalpy change upon unfolding; ΔS, entropy change; $\Delta G^{\#}_N$, free energy difference between ground state and transition state; E_a, activation energy; r, heating rate k_u, unfolding rate constant; k_f, (re-)folding rate constant; k_i, rate constant for the irreversible process; $t_{1/2}$, half-life time; f_N, fraction of folded protein; Mops, 3-(*N*-morpholino)propanesulfonic acid.

TABLE ONE

List of homologous α-amylases for which the apparent transition temperatures measured at 1 °C/min heating rate were determined under calcium-saturated and calcium-depleted conditions

Origin, α-amylase from	PDB entry	No. of residues	Molecular mass	No. of Ca ions	$T_{1/2}$	
					Calcium-saturated	Calcium-depleted
			kDa		℃	℃
Pig pancreatic (PPA)	1DHK	496	55.357	1	65	48
Aspergillus oryzae (TAKA)	6TAA	478	52.490	2	71	57
B. subtilis (BSUA)	1BAG	619	68.421	3	86	46
B. amyloliquefaciens (BAA)	1E43	483	58.843	4	86	38
B. licheniformis (BLA)	1BLI	483	58.274	3	102	51

lengths of 280 and 295 nm were applied, whereas emission spectra were recorded in the range between 300 and 450 nm. All fluorescence spectra were corrected for background scattering as measured with pure buffer. In some cases an UG1 filter (Schott, Mainz Germany) was used to attenuate the exciting beam, to avoid photo-bleaching and to obtain most reproducibly fluorescence emission spectra. Temperature scans were performed using a constant-temperature cuvette holder connected to an external constant temperature circulator/bath (F25, Julabo). Protein solutions (protein concentrations: 7 μg/ml to 1 mg/ml) were filled into quartz cuvettes with an optical path-length of 1 cm (104F-QS, Hellma). Temperatures of protein solutions up to ~110 °C were reached in sealed cuvettes without evaporation and boiling. Unfolding transitions were analyzed in terms of wavelength shifts of the emission peak and (or) by calculating the peak ratios of intensities as measured at 330 nm and 350 nm (at 345 and 335 nm for PPA, respectively).

Light Scattering—To estimate protein aggregation occurring upon thermal unfolding, an RF-1501 fluorospectrometer was employed to measure elastic scattering at 500 nm. For this purpose the same experimental setup (temperature-controlling unit; quartz cuvettes with 1-cm optical path-length) was used as for fluorescence studies.

Circular Dichroism—CD spectra in the far-UV region (200–280 nm) were recorded on a Jasco J-810 equipped with Peltier thermostatting cuvette holder under constant nitrogen flow. The spectra were recorded in a 0.2-cm cell at protein concentration of 0.05–0.1 mg/ml, averaged over three scans, and finally corrected for the buffer signal. The raw data were corrected for pre- and post-transition slopes. Thermal unfolding transitions were monitored by taking the CD signal at 222 nm as a function of temperature.

Kinetic Studies—Kinetics of thermal unfolding and of protein aggregation was measured using fluorescence or CD spectroscopy, and light scattering, respectively. For this purpose, buffer solutions were placed in the spectrometer for 5–10 min to reach thermal equilibrium at desired temperatures, before a small amount of concentrated protein solution was rapidly added during permanent stirring and data acquisition was started. The dead time of this procedure was about 10 s.

RESULTS

Homologous α-Amylases from Mesophilic Sources with Different Thermostabilities—As shown in TABLE ONE selected α-amylases exhibit rather different thermostabilities, despite the fact that all stem from mesophilic sources. The obtained apparent transition temperatures $T_{1/2}$ (with the amount of folded protein $N = N_0/2$ but not necessarily $N = U = 1/2$ as obtained from a reversible two-state unfolding process in equilibrium) were determined with fluorescence and CD spectroscopy by applying a heating rate of 1 °C per minute. All calcium-saturated and calcium-depleted samples (applying this heating rate) reveal rather similar transition temperatures with respect to already published data (13, 14, 19). To our knowledge, for BSUA no transition temperatures of thermal unfolding have been published yet. The well known effect of stabilization by calcium ions as part of all selected α-amylase structures (12, 14, 16), is much more pronounced for the more thermostable enzymes (21). For all techniques used to monitor the unfolding transition, such as CD and fluorescence spectroscopy, as well as differential scanning calorimetry, the same transition temperatures within the limits of error were obtained. Calcium saturated samples exhibit thermal unfolding transitions that appear as single steps with a more or less cooperative loss of secondary and tertiary structure. In the following studies presented here, only calcium saturated samples were used.

Effects of Aggregation and Heating Rates—For all α-amylases studied here the unfolding transitions were shown to be irreversible, not only for thermal unfolding but also for guanidine hydrochloride-induced unfolding (see also Refs. 11, 13, 14, and 22). It is supposed that chemical modifications, such as deamidation, cysteine oxidation, or peptide bond hydrolysis, take place once the protein is unfolded (1, 10). The most common model to describe this type of unfolding is given by Reaction 1,

$$N \underset{k_f}{\overset{k_u}{\longleftrightarrow}} U \xrightarrow{k_i} I$$

REACTION 1

where N is the native state, U is the unfolded state, and I represents the final irreversible denatured state, which quite often forms aggregates (see for example Refs. 6, 11, and 23). Whether irreversible processes have a significant impact on the apparent thermostability depends on several factors. Because irreversible processes, such as aggregation, are kinetic processes, the relation between transition rates and experimental heating rates plays an important role. Therefore, one of the main questions is: which of the two steps in the unfolding process, either the reversible unfolding (k_u) or the irreversible (k_i), is the rate-limiting step? In addition, due to the fact that aggregation is related to an interaction of at least two particles, the process depends on the protein concentration.

As shown in Fig. 1 all α-amylases exhibit a distinct heating rate dependence of apparent transition temperatures. Considering the limited number of only a few different heating rates, the relative differences in $T_{1/2}$ appear rather similar for all investigated enzymes. For practical reasons the heating rate dependence of transition temperatures have been fitted with a second-order polynomial function. Inspection of the fitting curves suggest that at heating rates above 3 °C per minute a maximal transition temperature is reached and no further increase of $T_{1/2}$ takes place. Although the course of the curves at low heating rates provides $T_{1/2}$ values at a heating rate zero (intercept with the y-axis), these

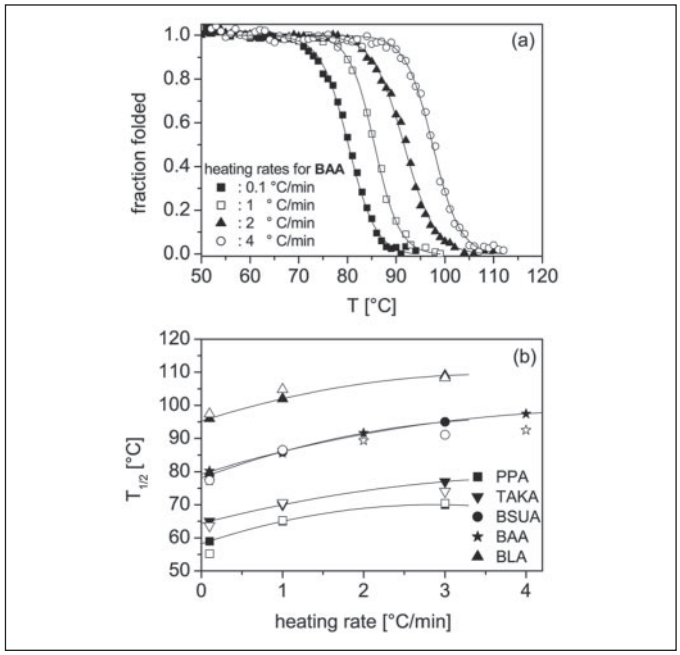

FIGURE 1. **Heating rate dependence of apparent transition temperatures (protein concentration: 50 μg/ml).** *a*, unfolding transitions of BAA as measured with CD spectroscopy are shown for four different heating rates. For all amylases the decrease of the fraction of folded protein was assumed to be proportional to the decrease of the CD signal (or alternatively of the fluorescence signal as in Fig. 2) with raising temperatures. The temperature dependence of the data was fitted with a sigmoidal Boltzmann function using Origin6.1 (OriginLab Corp., Northampton, MA). *b*, the obtained midpoint temperatures $T_{1/2}$ (*solid symbols*) as a function of the heating rate *r* were fitted applying $T_{1/2} = a + br + cr^2$. The *open symbols* represent corresponding $T_{1/2}$ values as calculated from kinetic data (see Equation 2).

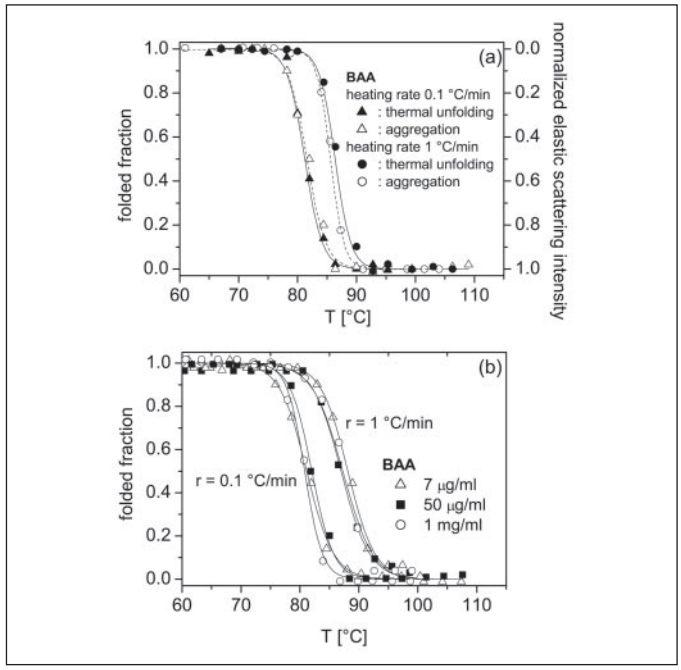

FIGURE 2. **Temperature scans for unfolding transitions of BAA as measured with fluorescence spectroscopy.** *a*, the accompanying protein aggregation was measured by elastic light scattering (*right legend* of the *y*-axis). For both heating rates the *solid lines* fit the fluorescence unfolding data, and the *dotted lines* fit the aggregation data (Boltzmann functions). The protein concentration was (50 μg/ml). *b*, temperature scans for three different protein concentrations are shown for the heating rates 0.1 and 1.0 °C/min.

values are meaningless. In the case of irreversible unfolding with much faster aggregation as compared with the unfolding transition (see below) the enzymes will unfold well below the $T_{1/2}$ (even though much slower), and an extrapolation to a heating rate of zero is not justified with the above given function.

With the exception of TAKA[3] all investigated enzymes show a pronounced aggregation upon thermal unfolding, which is already visible by eye due to turbidity of the protein solution. This phenomenon was analyzed in more detail by employing elastic light scattering, which is rather sensitive for aggregation if the particles increase in size from diameter $a < \lambda/20$ (dissolved monomeric proteins) to $a \approx \lambda$ (aggregates of proteins). Temperature scans of unfolding transitions and of the accompanying aggregation are shown for BAA in Fig. 2*a*. For both heating rates the aggregation appears rather concurrent with respect to the unfolding transition as obtained from tryptophan fluorescence. In addition unfolding transitions and aggregation were measured with different protein concentrations (7 μg/ml, 50 μg/ml, and 1 mg/ml; see Fig. 2*b*). Within the limits of error all protein concentrations exhibit the same apparent transition temperatures for the respective enzymes. A comparison between kinetics of the aggregation process and of unfolding kinetics (see next section Fig. 3*c*) revealed rather similar rates for both processes. On the basis of these observations we can conclude that the aggregation process 1) appears to be rather similar for all investigated α-amylases and 2) is very fast with respect to the unfolding transition ($k_i \gg k_u$). Furthermore, significant aggregation occurs only for fully (or partly) unfolded states but not for the native state under conditions used here. With respect to the kinetic model described in Reaction 1, our results indicate that the unfolding transition (determined by k_u) is the rate-limiting step.

Unfolding Kinetics—To characterize and to compare the individual thermostabilities of the given α-amylases we measured the temperature dependence of unfolding rate constants. As shown for the example of BAA (see Fig. 3) all unfolding transitions can be described very well with mono-exponential curve fitting. One approach to interpret the data is to assume an unfolding limited first-order kinetic process, which can be analyzed using the Arrhenius equation,

$$k = Ae^{-E_a/RT} \qquad \text{(Eq. 1)}$$

Applying this approach, activation energies (E_a) and pre-exponential factors (A) were obtained from the experimental kinetic rates k, whereas T is the absolute temperature and R the universal gas constant. Linear regression analysis applied to $\ln(k)$ *versus* $1/T$ plots (Arrhenius plots, see Fig. 4) yielded E_a (slopes of the curves) and A (y-axis intercepts), according to Equation 1 (see TABLE TWO for results). Due to the limitation of our experimental setup, we did not measure unfolding processes faster than ~50 s ($t_{1/2}$: half-life times). At least in one case (BAA) we measured rather slow unfolding processes at temperatures much below the apparent transition temperature as obtained at the lowest heating rate with $T_{1/2} = 80$ °C. In this case, BAA exhibits a half-life time of 5.13 h at 70 °C. Within the limits of error we observe for all α-amylases a linear behavior in the corresponding Arrhenius plots (Fig. 4). The obtained activation energies E_a (more precisely the enthalpic contribution to the activation energy) differs throughout the investigated enzymes. Although BLA exhibits the highest value of 363.7 kJmol^{-1}, BSUA and BAA show rather similar values around 260 kJmol^{-1}. A comparable large E_a value for TAKA (317.9 kJmol^{-1}) seems to be counterbalanced by a relatively large entropic contribution, which leads to a rather low thermostability of TAKA as compared with BAA and BSUA. The least thermostable PPA

[3] For unfolded TAKA only rather weak elastic scattering was observed. Nevertheless, TAKA unfolds irreversible and possibly the unfolded TAKA forms much smaller particles of protein aggregates as compared to the other α-amylases.

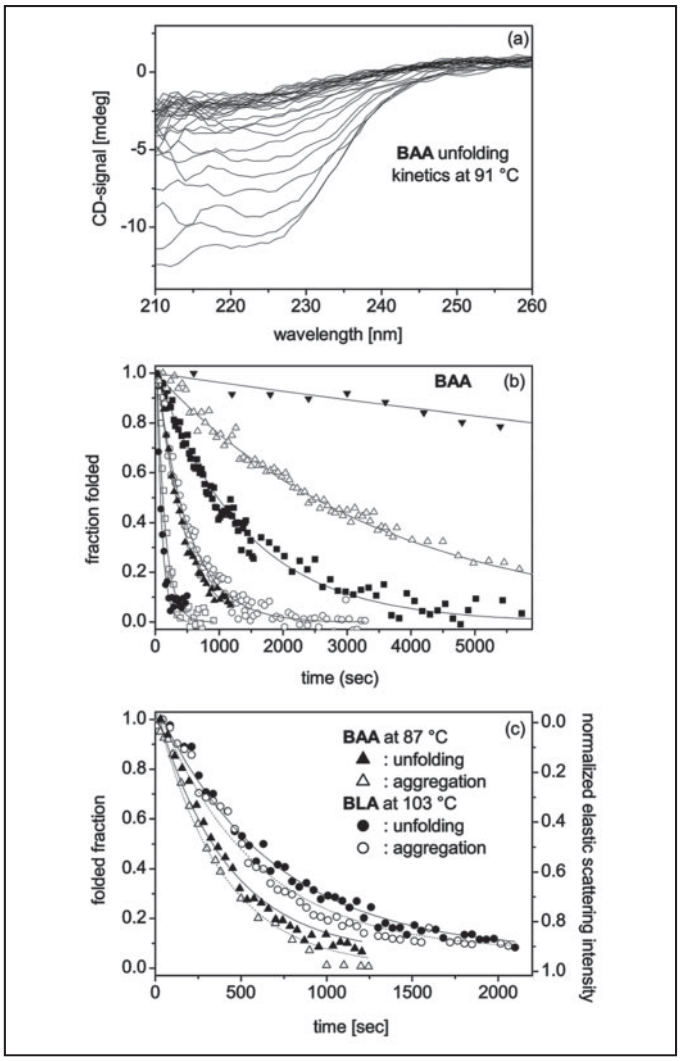

FIGURE 3. *a*, CD spectra as measured for BAA (50 μg/ml) at 91 °C in a time interval of 26 s. The decrease of the signal at 222 nm is assumed to be proportional to decrease of the fraction of folded protein with elapsing time. *b*, mono-exponential fits (*solid lines*) have been applied to experimental data points for the following temperatures (from slowest to fastest transitions): 70, 78, 82, 85, 87, 91, and 95 °C. For all amylases the experimental error of the obtained unfolding rates k_u (see Fig. 4) is in the order of ± 5–7%. *c*, as shown here for BAA and BLA (each 50 μg/ml) the kinetics of thermal unfolding and accompanying aggregation appear rather similar. A slightly faster aggregation process might be related to the fact that already partly unfolded proteins start to aggregate.

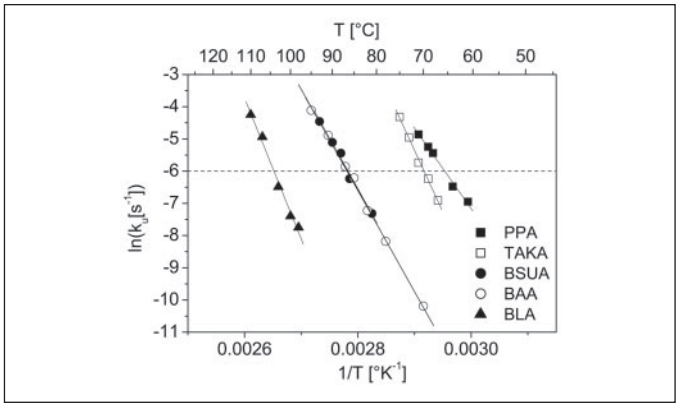

FIGURE 4. **Arrhenius plots for unfolding transitions of different α-amylase as measured with CD spectroscopy.** The *dotted line* represents unfolding rates, which are approximately related to values obtained for the apparent transition temperature $T_{1/2}$ at heating rates of 1 °C/min. These temperatures can easily be identified by projecting the intercept of the *dotted line* with the Arrhenius plots to the upper *y*-axis, which represents the corresponding temperatures. The results of linear regression analysis are given in TABLE TWO.

has the smallest E_a value of 207.5 kJmol^{-1} accompanied by the smallest entropic contribution. With respect to a reference temperature of 100 °C BLA exhibits a half-life time, which is approximately four orders of magnitude larger as compared with PPA and TAKA, and two orders of magnitude larger than those of BAA and BSUA. Because the E_a values for the α-amylases do not differ too much the relation of half-life times as obtained at 100 °C is rather similar to values determined at 80 °C. At even lower temperatures, of for example 60 °C, BLA shows already a half-life time that is already four orders of magnitude larger than BAA and BSUA and approximately six orders of magnitude larger with respect to those of PPA and TAKA. This indicates that with respect other α-amylases, BLA (and to a certain extend also TAKA) is kinetically more stabilized at lower temperatures as compared with higher temperatures. The half-life times obtained at the respective apparent transition temperatures (roughly indicated by the *dotted line* in Fig. 4) are rather similar, with a slightly larger value for BLA. A comparison of unfolding rates at the respective apparent transition temperatures strongly depends on the correct value of $T_{1/2}$, because small deviations in $T_{1/2}$ can lead to rather different unfolding rates. However, in principle this result suggests similar unfolding processes of the investigated α-amylases at their respective transition temperatures.

The consistency between scan-rate-dependent thermal unfolding data and the obtained unfolding rate coefficients can be checked easily by calculating the fraction of folded protein $f_N(T,r)$,

$$f_N(T,r) = \exp\left(-\frac{1}{r}\int_{T_0}^{T} A e^{-E_a/RT} dT \right) \qquad \text{(Eq. 2)}$$

where T_0 denotes a temperature at which the fraction of unfolded proteins is negligible, and T is the temperature of interest (for more details see Refs. 8, 25, and 26). Values as obtained using Equation 2 were fitted with sigmoidal Boltzmann functions, and the resulting transition temperatures ($T_{1/2}$) are shown in Fig. 1*b* (*open symbols*). The comparison with scan-rate data (*solid symbols* this figure) yields some systematic deviations at higher heating rates for BAA, BSUA, and TAKA. This discrepancy may be due to the fact that a temperature lag in the cuvettes relative to the heating system had occurred. However, considering the experimental error of fundamental parameter k_u (±5–7%) the obtained consistency appears satisfactory.

DISCUSSION

The impact of aggregation and heating rates on the apparent transition temperatures has been described for several cases in previous studies (6–8, 23). Although it is quite often stated that heating rates of 1 °C per minute are reasonable to measure the unfolding transition of mesophilic proteins, it was demonstrated in various studies that equilibrium was not reached. There are many possible reasons for such an observation. In some cases slower heating rates are required due to unusually slow relaxation unfolding or refolding kinetics (27, 28). In other cases the irreversible process leading to aggregates is rate-limiting and therefore high heating rates can reduce irreversible transitions (7, 8). In this case one can even obtain thermodynamic equilibrium parameters from irreversible unfolding transitions (7). In contrast we observe for all α-amylases an irreversible process, which is fast as compared with the unfolding transition. This seems to be the more common case for most multidomain proteins. In this case equilibrium thermodynamics is without any importance for the thermostability. Only the barrier height

TABLE TWO

Parameter obtained from linear regression fits as shown in Fig. 4

The experimental errors for the obtained unfolding rates and half-life times are in the order of ± 5–6%.

	E_a	k_u at 100 °C	$t_{1/2}$ at 100 °C	$t_{1/2}$ at $T_{1/2}$ h.r. 1 °C/min
	kJ mol^{-1}	s^{-1}	*s*	*s*
PPA	207.5 (±10.4)	2.25	0.308	314
TAKA	317.9 (±11.6)	19.96	0.035	267
BSUA	262.0 (±15.5)	5.337×10^{-2}	12.9	308
BAA	255.4 (±13.9)	5.429×10^{-2}	12.8	317
BLA	363.7 (±21.3)	6.929×10^{-4}	1000.4	535

of the transition state $\Delta G^{\#}_N$ as measured by k_u determines the thermostability. A comparable effect of kinetic stability caused by a remarkable high unfolding barrier was observed for a mechanism of protein longevity (29).

With respect to our kinetic model (Reaction 1) it is of interest whether a reduction (or prolongation) of the irreversible process would increase the apparent thermostability (30). One approach to reduce protein aggregation of unfolded α-amylases is to add co-solvents such as non-detergent sulfobetaine (31) or cyclodextrins (32). Based on thermal unfolding studies we observed for non-detergent sulfobetaine only a slight increase of the apparent transition temperature, but still no reversibility of the unfolding process (data not shown here). On the other hand, for chemical-induced unfolding (by guanidine hydrochloride) in some cases reversibility of the unfolding transition was observed (32, 33). Based on early observation by Tomazic and co-worker (10) the irreversibility of the thermal unfolding transition (at least for BLA) is caused by deamidation of amide residues at elevated temperatures. In this respect aggregation is only an accompanying process but is not the reason for the irreversibility. This appears to be rather different in the case of guanidine hydrochloride-induced unfolding. However, the irreversible thermal unfolding seems not to be an inherent property of larger multidomain proteins (even not at extreme high temperatures). As known from hyperthermophiles, a nascent polypeptide chain is able to fold into its native conformation at rather elevated temperatures. Therefore, it is still a question whether *in vitro* conditions can be found for reversible thermal unfolding or whether the help of other factors (*e.g.* molecular chaperons) as supplied under *in vivo* conditions are necessary (34).

In addition to a vast body of work on the kinetics of thermoinactivation, numerous studies on the unfolding kinetics of proteins have recently been published (34–42). In particular a comparison between mesophilic and thermophilic proteins was the focus of these studies. Besides some exceptions (40, 42), most studies revealed significant slower unfolding rates for thermophilic proteins as compared with their mesophilic counterparts. At a reference temperature of 100 °C for rubredoxin a difference in unfolding rates of 2–3 orders of magnitude was obtained (35). For glycosidases and peptidases at a much lower reference temperature (60–65 °C) differences of about five orders of magnitude in the unfolding rates were observed (37, 39). Although our comparison considers only mesophilic proteins the difference in unfolding rates between BLA and PPA (or TAKA) we obtained here are at least as large (if not even larger at the corresponding reference temperatures) as those obtained for the mesophilic-thermophilic pairs. Even for BAA (or BSUA) compared with PPA (TAKA) we obtained differences in unfolding rates of two orders of magnitude. Our results emphasizes that BLA is one of the most thermostable proteins from a mesophilic source (43). With respect to difference in unfolding kinetics between BLA and BBA, early studies on thermoinactivation (at 90 °C) revealed similar differences for these enzymes (11).

We have demonstrated, for irreversible unfolding α-amylases, that the obtained unfolding rate constants are very meaningful parameters to compare thermostabilities of different proteins. Similar to numerous thermophilic proteins, which exhibit very slow unfolding processes, we obtained extremely small unfolding rates for the thermostable BLA originating from a mesophilic source. In contrast to reversible unfolding proteins, where either kinetic or thermodynamic stability can be the key feature in thermostabilization (40), only kinetic barriers determine thermostability for the α-amylases with a fast irreversible process. Based on this, mutant studies often revealed enhanced stabilities that are determined kinetically rather than thermodynamically. For example, recently a BLA mutant was studied that unfolds 32 times slower than the wild type (44).

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