New light on protein folding: Unraveling folding and unfolding mechanisms using time-resolved and two-dimensional vibrational spectroscopy
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Salt bridges are known to play a critical role in the conformational stability of many proteins, but their impact on the kinetics of protein folding remains largely unresolved. In the previous chapter we have shown that Glu$^-$/Arg$^+$ salt bridges have a strong impact on the folding and unfolding kinetics of different $\alpha$-helical peptides, and can both speed up and slow down the rate of $\alpha$-helix formation. In this chapter, we extend this investigation and systematically study the kinetic effect of salt bridges formed between different types of charged amino-acid residue pairs, using combined temperature-jump transient-infrared measurements, steady-state UV circular dichroism, and molecular dynamics simulations. We determine the folding and unfolding rates of twelve alanine-based $\alpha$-helical peptides, each of which has a nearly identical composition containing three pairs of positively and negatively charged residues (either Glu$^-$/Arg$^+$, Asp$^-$/Arg$^+$, or Glu$^-$/Lys$^+$). Within each set of peptides, the distance and order of the oppositely charged residues in the peptide sequence differ, such that they have different capabilities of forming salt bridges. Our results indicate that geometrically optimized salt bridges (in which the interacting residues are spaced four peptide units apart, and their order is such that the side-chain rotameric preferences favor salt-bridging) speed up $\alpha$-helix formation, and slow down the unfolding of the $\alpha$-helix. On the contrary, salt bridges with an unfavorable geometry have exactly the opposite effect. Comparing the kinetic behavior of peptides with different types of charge pairs, we observe that a salt bridge formed between the rotamERICally optimized side chains of Glu$^-$ and Arg$^+$ is most favorable for the speed of folding, probably because of the larger conformational space of the salt-bridging Glu$^-$/Arg$^+$ rotamer pairs compared to Asp$^-$/Arg$^+$ and Glu$^-$/Lys$^+$. Interestingly, we find that the ac-
celerating effect of salt bridges on the folding rate occurs predominantly through the entropic part of the free-energy barrier (the enthalpic part even works in the opposite direction), suggesting that the salt bridges act as a folding nucleus. The observed impact of salt bridges on the folding kinetics provides a possible evolutionary explanation for the presence of salt bridges that do not stabilize the folded structure in many biologically active proteins: such apparently non-functional salt bridges might have a kinetic function and guide the protein towards a specific folding pathway, rather than having a static, structure-stabilizing function.

4.1 Introduction

The well-defined three-dimensional structure of folded proteins and peptides is determined by a large number of relatively weak noncovalent interactions, including hydrophobic effects, Van der Waals forces, hydrogen bonding, and electrostatic interactions [1]. The delicate balance between each of these noncovalent forces determines the overall thermodynamic stability of native protein folds, and their quantitative contribution is under ongoing discussion. Considerable evidence exists indicating that hydrophobic interactions are the major contributors to protein stability and folding [2], whereas Van der Waals interactions [3] and hydrogen bonding [4, 5] are believed to have relatively less energetic significance. The influence of electrostatic interactions between oppositely charged residues (salt bridges) on protein stability has proven to be particularly ambiguous [6–8]. This is because the impact of salt bridges on protein structure and stability depends strongly on the relative location of the salt-bridging residues in the primary sequence, the relative orientation and distance between the interacting residues, the effect of neighboring residues, and the degree of solvent accessibility [9–11]. Because the energetically favorable Coulomb interaction between salt-bridging amino-acid residues requires structural re-ordering and desolvation processes that might be energetically unfavorable [6], the net contribution of a salt bridge to the free-energy balance of proteins can range from highly stabilizing to highly destabilizing [6, 9, 12–25]. The latter type of salt bridges, that do not stabilize the folded state, must have a biological function of a different nature. In the previous chapter we found evidence that suggests that such apparently non-functional salt bridges might have a kinetic rather than a thermodynamic function [26].

The potential role of salt-bridge interactions in the kinetics of protein folding becomes increasingly relevant as MD evidence indicates that salt-bridging side chains can act as nucleation sites for the intramolecular folding process of amyloids [28]. However, whereas the thermodynamic effects of salt bridges have been extensively studied, [6–10, 12–23, 25] the exact role of salt bridges in the protein folding process remains controversial [29–40]. In the previous chapter we have shown that Glu−/Arg+ salt bridges have a strong impact on the kinetics of the helix-coil transition, and can both speed up and slow down the folding rate, depending on their geometry [26]. To gain further understanding into the effect of salt bridges involving different type of oppositely charged amino acid residues on
the kinetics of folding processes, in this chapter we investigate the kinetic role of
salt bridges formed between different charge pairs, viz., Glu−/Arg+, Asp−/Arg+, or Glu−/Lys+. To this purpose, we determine the folding and unfolding rates of twelve different alanine-based α-helical peptides (see Table 4.1), each of which has a nearly identical amino-acid composition with three repeating salt-bridge forming residue pairs (either Glu−/Arg+, Asp−/Arg+, or Glu−/Lys+). Within each set of different salt-bridge combinations, four different sequence patterns were studied that only differ in the relative spacing and geometric orientation of the oppositely charged residues, such that they have different capabilities of forming salt bridges (Fig. 4.1). Combined temporal and structural resolution was achieved

Figure 4.1: Schematic representation of the folded conformation of six of the investigated peptides, showing the salt-bridge forming side-groups [(a,b) Glu− (E) and Arg+ (R), (c,d) Asp− (D) and Arg+ (R), and (e,f) Glu− (E) and Lys+ (K)]. Left: the salt-bridging side-chains are spaced four \((i, i + 4)\) peptide units apart: peptides \((i + 4)\)ER (top), \((i + 4)\)DR (centre), and \((i + 4)\)KE (bottom). Right: the salt-bridging side-chains are spaced three \((i, i + 3)\) peptide units apart and are in the reversed order: peptides \((i + 3)\)RE (top), \((i + 3)\)RD (centre), and \((i + 3)\)KE (bottom). Pictures optimized and rendered with Chimera [27].
using transient-infrared (IR) measurements following a laser-induced temperature-jump (T-jump) \([41–47]\) in the amide I’ spectral region, which is a sensitive probe of the backbone conformation (see section 1.3.1). Combined analysis of our steady-state UV circular dichroism (UV-CD) and T-jump transient-IR measurements allows a detailed study of the folding and unfolding kinetics. Complementary to the experiments, molecular dynamics (MD) simulations have been carried out to obtain more insight into the correlation between salt-bridge population and \(\alpha\)-helical content, and into the order and distance dependence of the charged residue pairs on their capability to form a salt bridge.

### 4.2 Results and discussion

#### 4.2.1 Sequence design

The sequences of the investigated alanine-based \(\alpha\)-helical peptides (see Table 4.1) are based on a previously reported design \([12, 18]\). The peptides are 17 residues long and have nearly identical amino-acid compositions with three repeating salt-bridging residue pairs, viz., Glu\(^-\) (E) and Arg\(^+\) (R), Asp\(^-\) (D) and Arg\(^+\) (R), or Glu\(^-\) (E) and Lys\(^+\) (K). Within each set of salt-bridge combinations, the charged residues are positioned either three \((i, i + 3)\) or four \((i, i + 4)\) peptide units apart, and the different acidic (A, either Glu\(^-\) or Asp\(^-\)) and basic (B, either Arg\(^+\) or Lys\(^+\)) residues are ordered in the sequence as AB or BA (see Fig. 4.1). To avoid end-charge effects, the N- and C-terminals are blocked with acetyl and amide groups, respectively. To specifically examine the helix-stabilizing or destabilizing effect of the salt bridge and its impact on the folding and unfolding kinetics of the \(\alpha\)-helix to random-coil transition, each of the peptides was studied under both neutral and acidic pH conditions. At neutral pH, both the acidic and basic side chains are charged, and formation of an AB- or BA-oriented salt bridge is possible, whereas at low pH (pH \(\leq 2.5\)) the acidic side-groups are neutral (isolated Glu and Asp sidegroup \(pK_a\)’s are approximately 4.4 and 4.0, respectively) \([1]\), making salt-bridge formation impossible. We verify that the side-chains of the Glu or Asp residues are completely protonated from the IR response of the COO\(^-\) (Glu\(^-\) 1565 cm\(^{-1}\), Asp\(^-\) 1586 cm\(^{-1}\)) and COOH (Glu 1705 cm\(^{-1}\), Asp 1713 cm\(^{-1}\)) groups (see Fig. 4.12) \([48]\).

#### 4.2.2 Equilibrium properties using UV-CD

The unfolding transition of the investigated peptides was studied in thermal equilibrium by collecting temperature-dependent UV-CD spectra between 190 to 257.5 nm (Fig. 4.11) under both neutral (salt-bridge effects) and acidic (no salt bridges) pH conditions. Fig. 4.2 shows the temperature dependence of the mean residue ellipticity detected at 222 nm for each of the peptides at neutral and acidic pH. All melting curves show a sigmoidal transition, reflecting the temperature-induced conformational changes of the peptides in thermal equilibrium. From a singular value decomposition (SVD) of the temperature-dependent CD spectra...
and global fitting, we find that the α-helix to random-coil transition can be very well described by a two-state model. The melting temperatures ($T_m$) and the unfolding enthalpy changes ($\Delta H$) were obtained from least-square fits to a two-state model (see eq. 2.2) [49] and are summarized in Table 4.2.

As we have shown in the previous chapter (see section 3.4.3), the relative

![Thermal denaturation curves](image)

**Figure 4.2:** Thermal denaturation curves at 222 nm using UV-CD of the different E/R peptides (top), D/R peptides (centre), and E/K peptides (bottom), under neutral (left) and acidic (right) pH conditions. The solid curves represent least-square fits to a two-state unfolding transition (eq. 2.2) [49]. The experimental values obtained for $\Delta H$ and $T_m$ are listed in Table 4.2.
helicity and thermodynamic stability of the Glu/Arg peptides at neutral pH follows the trend \((i + 4)ER > (i + 3)ER \approx (i + 4)RE > (i + 3)RE\), indicating that an ER-oriented salt bridge with E and R spaced four peptide units apart is most favorable for the folded, \(\alpha\)-helical conformation (see Fig. 4.2 and Table 4.2). A similar trend is observed for the D/R salt bridges, viz., \((i + 4)DR > (i + 3)DR \approx (i + 4)RD \approx (i + 3)RD\), in agreement with previous results [18]. Note that the UV-CD spectrum of peptide \((i + 3)RD\) indicates that at neutral pH the random-coil conformation is predominantly populated even at the lowest temperatures (see Fig. 4.11), making a quantitative determination of the thermodynamic parameters impossible. The conformational stability of the peptides containing E/K salt bridges follows a trend analogous to the Arg-based peptides, viz. \((i + 4)EK > (i + 3)EK \approx (i + 4)KE > (i + 3)KE\), suggesting that \((i + 4)EK\) is the most favorable Lys-based salt-bridge geometry in an \(\alpha\)-helix. Thus, within each particular set of E/R, D/R, and E/K salt bridges we observe a similar sequence-distance and order dependence, and find that the \(\alpha\)-helix-stabilizing effect is largest for AB-oriented salt bridges in which the interacting side chains are spaced four \((i, i + 4)\) peptide units apart, whereas the \((i + 3)BA\) type of salt bridges are less favorable for the \(\alpha\)-helical conformation. This behavior probably arises mainly from sterical effects due to the intrinsically preferred rotameric states of the interacting amino-acid residues [50–55], and will be discussed below.

A comparison of helix content and thermodynamic stability between peptides containing different types of salt bridges (either E/R, D/R, or E/K) reveals significant differences (see Fig. 4.3a). We find that the thermodynamic stability of the \(\alpha\)-helices with optimized salt bridges follows the trend \((i + 4)ER > (i + 4)DR \approx (i + 4)EK\), suggesting that substitution of the Glu residues with Asp, or Arg residues with Lys, leads to destabilization of the \(\alpha\)-helical conformation (see Fig. 4.2 and Table 4.2). The observed differences in conformational stability between peptides containing different charge pairs possibly arises from the intrinsic helix-forming propensity of the specific amino acids [56]. An additional explanation is that the rotameric accessibility of salt bridges between the charged side chains in an \(\alpha\)-helix may differ depending on the type of amino acid residue pairs (either Glu/Arg, Asp/Arg, or Glu/Lys), possibly causing differences in their capabilities of forming salt bridges and thereby in the thermodynamic stability of the \(\alpha\)-helix. In particular, the number of possible low-energy rotamer pairs that allow salt-bridge formation can cause a difference in the helix-stabilizing effect of the salt bridge, because the restriction of a salt-bridge to a single rotameric state in the helical structure carries a significant entropic penalty [51, 52], whereas interconversion between multiple salt-bridging rotameric states (through isomerization along different \(\chi\) angles) can reduce the entropic cost of salt-bridge formation [53, 54]. We observe that the Arg-based peptides that contain Asp in all cases have a lower thermodynamic stability than the corresponding peptides with Glu. Due the shorter chain length of Asp, substitution of the Glu residues with Asp changes the positional distribution of the carboxylate groups, thereby possibly limiting the number of probable low-energy conformations that allow salt-bridge formation and resulting in a reduced stability of the \(\alpha\)-helix. Furthermore, we find that re-
placement of Arg with Lys also destabilizes the α-helical conformation of peptides (i + 4)ER and (i + 3)ER. Compared to the guanidinium moiety of the Arg side chain, the single amino group of Lys allows a reduced number of potential interactions with the carboxylate of Glu, resulting in different geometric specificities of Glu-Lys and Glu-Arg salt bridges [55, 57], and possibly in a different rotamer side-chain distribution favoring salt-bridge interactions with Glu. The number of AB-oriented salt-bridge forming Glu-Lys rotamer pairs in an α-helical backbone conformation might be less compared to Glu-Arg, causing a decreased stability of the folded state of the peptides with EK-oriented salt bridges compared to their Arg-based analogs. In contrast, a comparison of the thermodynamic stability of the KE- and RE-oriented peptides indicates that these geometric effects become smaller upon reversing the charge orientation. To obtain more detailed insight into the folded and unfolded conformations and the relative salt-bridge populations of the investigated peptides we have carried out MD simulations (see section 4.2.3).

To specifically determine whether a particular salt-bridge type provides (de)stabilization to the α-helical conformation of the different peptides, the contribution of salt-bridge interactions was eliminated by protonating the carboxylates of either Glu or Asp at acidic pH (Fig. 4.2, right side). As shown in the previous chapter (section 3.4.3), the relative helicity and thermodynamic stability of the Glu/Arg peptides at acidic pH follows the trend (i + 4)ER ≈ (i + 4)RE > (i + 3)ER ≈ (i + 3)RE. The dichotomy observed between the (i, i + 4) and (i, i + 3) spaced Glu/Arg peptides in the absence of salt bridge interactions probably arises from the slight positional dependence of the intrinsic helix-forming tendencies of some amino acids [58]. For the Asp/Arg peptides, we find that the low-temperature UV-CD spectra of peptides (i + 3)DR, (i + 4)RD, and (i + 3)RD exhibit essentially no helicity at acidic pH (see Fig. 4.11), and the thermodynamic parameters for these three peptides could therefore not be reliably determined. However, the rel-
ative helicity at acidic pH follows the trend $(i + 4)\text{DR} > (i + 3)\text{DR} \approx (i + 4)\text{RD} > (i + 3)\text{RD}$, in agreement with previous results [18]. The thermodynamic-stability trend of the Glu/Lys peptides in the absence of salt-bridge interactions is $(i + 4)\text{EK} \approx (i + 4)\text{KE} \approx (i + 3)\text{KE} \approx (i + 3)\text{EK}$, indicating no significant positional dependence of the Lys residues at acidic pH. Overall, within each set of salt-bridge combinations we find that the $\alpha$-helical content and thermodynamic stability of the AB-oriented peptides decrease in the absence of salt-bridge interactions. By contrast, all BA-type peptides (except for peptide $(i + 4)\text{RD}$) show an enhanced helicity and/or stability upon salt-bridge breaking, indicating that the formation of BA-oriented salt bridges at neutral pH destabilizes the folded conformation. Comparison of the melting curves of the different sets of E/R, D/R, and E/K peptides obtained at neutral (Fig. 4.2, left column) and acidic pH (Fig. 4.2, right column) shows that the $\alpha$-helical content and thermal stability of the different E/R, D/R, and E/K peptides exhibit different degrees of pH dependence. The helicity and stability of the best helix-forming peptides from each set of E/R, D/R, and E/K sequences at acidic pH follows the trend $(i + 4)\text{ER} > (i + 4)\text{EK} \gg (i + 4)\text{DR}$, so that the relative pH dependence is largest for the Arg-based peptides containing Asp, and smallest for the Lys-based peptides.

### 4.2.3 Analysis of salt-bridge formation using MD simulations

To study the equilibrium properties of the investigated peptides in more detail, we performed six 300 ns Molecular Dynamics (MD) simulations for each peptide, starting from a helical structure and different velocities for each run. As a measure for the stability of the helical conformation, we counted the number of backbone hydrogen bonds between the carbonyl oxygen $O$ in residues $i$ and the N-H group in residues $i + 4$, indicated as $\langle h_{\text{hhb}} \rangle$. In a perfect helix, this number is 15, including the caps on the termini. Fig. 4.14 in section 4.5.3 shows the six time traces of $\langle h_{\text{hhb}} \rangle$ for each peptide. Most systems lose some, or all of their helical structure during the simulations. In some systems, such as for example $(i + 4)\text{ER}$ and $(i + 4)\text{EK}$, show unfolding and refolding within 150 ns. Therefore, we assume that after 150 ns all systems have lost memory of the starting conditions, and subsequent analysis includes only data from the last 150 ns.

Fig. 4.4 (left panels) show the probability distribution of the number of helical hydrogen bonds for each peptide, with the mean indicated by a dashed line. Overall, the peptides with the negative charge preceding the positive charge in the sequence show the highest values for $\langle h_{\text{hhb}} \rangle$, with $(i + 4)\text{DR}$ as the most stable peptide ($\langle h_{\text{hhb}} \rangle = 8$). The Glu-Lys peptides show the lowest values for $\langle h_{\text{hhb}} \rangle$, with peaks at $\langle h_{\text{hhb}} \rangle = 0$ for $(i + 3)\text{EK}$, $(i + 4)\text{KE}$ and most pronounced for $(i + 3)\text{KE}$. With the value of $\langle h_{\text{hhb}} \rangle$ indicating the helical stability of the peptide, the order of peptides from most stable to least stable is for the Glu-Arg based peptides $(i + 4)\text{ER} \approx (i + 3)\text{ER} > (i + 4)\text{RE} \approx (i + 3)\text{RE}$, for the Asp-Arg series $(i + 4)\text{DR} > (i + 3)\text{RD} > (i + 3)\text{DR} \approx (i + 4)\text{RD}$ and for the Glu-Lys series $(i + 4)\text{EK} > (i + 3)\text{EK} \approx (i + 4)\text{KE} \approx (i + 3)\text{KE}$. In all three series, the peptides with the acidic group positioned at the N-terminal side of the repeat retain more helical structure.
than the peptides with the basic group positioned first in the repeat. This is in excellent agreement with the experimental observations.

For each helical hydrogen bond we calculated the distance between the donor (N) and the acceptor (O) $d_{O-N}$. In Fig. 4.4 (right panels), $d_{O-N}$, averaged over the six simulations (excluding the first 150 ns) are plotted as function of the residue number of the acceptor. The dashed line at 0.35 nm indicates the distance criterion for forming a hydrogen bond. In the Glu-Arg and Asp-Arg series, and for $(i+4)EK$, $d_{O-N}$ is highest at the ends of the peptide, indicating fraying of the $\alpha$-helix. Even though $(i+4)RE$ and $(i+3)RE$ have the same $\langle hhb \rangle$, the latter has a less stable C-terminal side, while $(i+3)RE$ has a less stable N-terminal side. In the Glu-Lys series, only $(i+4)EK$ contains intact helical hydrogen bonds in all simulations. Intact helical hydrogen bonds do occur for the three other Glu-Lys peptides but only in one or two runs, as demonstrated in the helical hydrogen bond time traces in section 4.5.3.

Salt bridges may form within the same repeat, but they also form across repeats. We calculated the fractions of salt bridges formed within the same repeat, across different repeats, or not formed at all. Fig. 4.5 shows the fraction of the

**Figure 4.4:** Helical hydrogen bonds (hhb) in the peptide systems. Left: the probability distribution ($p$) of the number of hhb for each peptide, with the mean indicated by a dashed line. Right: the distance between the donor (N) and the acceptor (O) $d_{O-N}$, averaged over the six simulations (excluding the first 150 ns) as function of the residue number of the acceptor. The dashed line at 0.35 nm indicates the distance criterion for forming a hydrogen bond.
different types of salt bridges present in the simulations. Overall, the fraction of formed salt bridges is larger for the peptides with the acidic group occurring first in the repeat. Moreover, these peptides hardly show any salt bridges formed across repeats, with the exception of \((i+4)ER\) and \((i+3)EK\). With the basic group occurring first in the repeat, the fraction of salt bridges formed across repeats is larger.

To investigate the effect of the salt bridges on the stability of the helices, we calculated two-dimensional probability distributions for \(\langle hhb \rangle\) and the number of intact salt bridges, formed within the same repeat, \(nsb\), plotted in Fig. 4.6. In the peptides with the acidic group occurring first in the repeat \((i+4)ER, (i+4)DR, (i+4)EK\) and \((i+3)EK\) \(hhb\) and \(nsb\) show a positive correlation. Configurations with high helical content contain two or more in-repeat salt bridges, whereas conformations with little helical structure contain one in-repeat salt bridge at most. This indicates that the formation of salt bridges coincides with higher helical content. In contrast, peptides \((i+4)RE\) and \((i+3)RD\)

![Figure 4.5: Fraction of different salt-bridge types. The fraction of salt bridges is plotted as bar plots for each repeat, with black indicating in-repeat salt bridges, red and blue indicating out-repeat blue salt bridges, with red indicating a negatively charged residue being involved and blue a positively charged residue. Grey indicates no salt bridge has been formed.](image-url)
show a negative correlation between $hhb$ and $nsb$, revealing helical conformations without any formed salt bridges, or salt bridges formed in conformations with very little helical structure. Finally, the probability maxima in the profiles of peptides $(i+3)RE$, $(i+4)RD$, $(i+4)KE$ and $(i+3)KE$ contain little helical structure, and at most one formed salt bridge. For these peptides, no correlation between $hhb$ and $nsb$ is observed.

### 4.2.4 Infrared spectra

In the kinetic studies of the $\alpha$-helices, we use the IR response as a probe of conformation. We first probe the $\alpha$-helix to random-coil transition in thermal equilibrium. Temperature-dependent FTIR spectra were collected in the amide I’ spectral region. The amide I’ vibration of proteins and peptides, primarily arising from carbonyl stretch vibrations of the backbone amide residues, is very sensitive to secondary structure and can be directly related to the conformation of the backbone [59]. In particular, the IR absorption band of amide residues in an $\alpha$-helical structure is centered at $\sim 1635 \text{ cm}^{-1}$, whereas a random backbone conformation absorbs at higher frequencies ($\sim 1645 \text{ cm}^{-1}$) [48, 60]. To characterize the
temperature-induced absorption changes of the amide I’ mode, equilibrium FTIR spectra were collected between 274 K and 353 K with incremental steps of 5 K. As can be seen in Fig. 4.7, all low-temperature FTIR spectra (blue) of the \((i, i+4)\)-spaced and AB-oriented peptides exhibit an amide I’ band centered at 1634 cm\(^{-1}\) arising from the amide carbonyl groups involved in an \(\alpha\)-helical conformation. With increasing temperature (Fig. 4.7, red), the amide I’ absorption maxima are shifted from 1634 cm\(^{-1}\) to 1644 cm\(^{-1}\), indicative of an \(\alpha\)-helical to random-coil transition. Accordingly, all temperature-difference spectra show a broad negative feature centered at 1630 cm\(^{-1}\) and a positive feature centered at 1658 cm\(^{-1}\) (Fig. 4.7, green). The negative contribution is characteristic for the loss of \(\alpha\)-helical population, whereas the positive feature is attributed to the associated formation of random-coil conformations [44]. In addition, the minor components occurring at 1585 and 1607 cm\(^{-1}\) in the FTIR spectra of the Arg-containing peptides \((i+4)\)ER and \((i+4)\)DR (Fig. 4.7 a,b), arise from the symmetric and antisymmetric stretch vibration of the guanidinium (CN\(_3\)H\(_5^+\)) group of Arg [48]. The small peak at 1565 cm\(^{-1}\) in the spectra of the Glu-based peptides \((i+4)\)ER and \((i+4)\)EK (Fig. 4.7 a,c), originates from side-chain contributions of the carboxylate group of Glu\(^-\), and shifts to 1705 cm\(^{-1}\) upon protonation under acidic pH conditions (Fig. 4.12 a,c) [48]. As can be seen in Fig. 4.7b, the CO-stretching mode of the COO\(^-\) group of Asp\(^-\) arises at 1586 cm\(^{-1}\), and shifts to 1713 cm\(^{-1}\) upon protonation (Fig. 4.12b) [48]. The absence of the COO\(^-\) peaks in the FTIR spectra obtained at low pH confirms that the carboxylate groups of the Glu and Asp side chains are completely protonated under acidic pH conditions, thereby inhibiting the formation of salt bridges (Fig. 4.12 b,d,e).

**Figure 4.7:** Temperature-dependent equilibrium FTIR spectra in the amide I’ spectral region of peptides (a) \((i+4)\)ER, (b) \((i+4)\)DR, and (c) \((i+4)\)EK at neutral pH. FTIR difference spectra (green) were generated by subtracting the spectrum measured at 274 K (blue) from the spectrum collected at 353 K (red), and reflect the changes in IR absorption upon thermal unfolding of the \(\alpha\)-helices. pH\(^*\) = uncorrected pH meter reading in D\(_2\)O.
4.2 Results and discussion

4.2.5 T-jump relaxation kinetics

We probed the T-jump-induced relaxation kinetics of each of the investigated α-helices in the amide I’ spectral region starting from various initial temperatures. Fig. 4.8a shows a solvent-corrected three-dimensional representation of the relaxation kinetics of peptide (i + 4)EK at neutral pH in response to a T-jump from 293 to 301 K. The time- and frequency-resolved change in IR absorption ($\Delta A$) shows negative (blue) and positive (red) contributions reflecting the blue shift of the amide I’ mode that arises from the conformational redistribution process of the peptide during re-equilibration. As can be seen in Fig. 4.8b, the transient spectra at long time delays are essentially identical to the equilibrium FTIR temperature-difference spectrum and exhibit the negative and positive contributions at 1630 and 1658 cm$^{-1}$, indicative of an α-helix to random-coil transition. We find that the T-jump relaxation kinetics probed at these two structure-sensitive frequencies can be well described by a single-exponential function, i.e. with an observed relaxation time constant of $\tau = 143 \pm 4$ ns at a final temperature of 301 K (Fig. 4.8c). Moreover, for each of the investigated peptides we find that the transients show single-exponential T-jump relaxation kinetics with equal relaxation rates at 1630 cm$^{-1}$ (increase of the α-helical population) and 1658 cm$^{-1}$ (increase of the random-coil population), at all final T-jump temperatures, and at all pH values.

The detection of single-exponential T-jump relaxation kinetics at all final temperatures, IR frequencies, and pH values, indicates that the folding and unfolding pathways of the investigated α-helical peptides can be represented by an effective two-state model, [61] as already suggested by our static temperature-dependent measurements. While classifying the conformational population distribution of α-helices into two macroscopically distinct states, viz., ‘α-helix’ and ‘random-coil’, might not be rigorously correct, effective two-state folding behavior has been observed previously for a large number of α-helices [38, 62–68]. These experimental observations probably originate from a conformational free-energy landscape in which the α-helical and random coil ensembles can be characterized as two broad free-energy minima that are separated by a single main free-energy barrier [63–65]. When the free-energy barrier separating these two conformational macrostates is much higher than the barriers between the microstates within each of them, pre-equilibration within the microstate populations occurs on timescales much faster then the population exchange involving the crossing of the main free-energy barrier [69–72]. If the population distribution of all microstates pre-equilibrate within the time-resolution of the T-jump pulse (~5 ns), their relative populations will retain constant from this point in time, and single-exponential T-jump relaxation kinetics reflecting the crossing of the main free-energy barrier and the conformational re-distribution process between the α-helical and random-coil ensembles will be observed [71]. Accordingly, the detection of a single kinetic timescale indicates that the investigated α-helical peptides fold and unfold effectively as two-state systems, allowing the estimation of the effective folding and unfolding rates ($k_F$ and $k_U$) involving the crossing of the main free-energy barrier using a combined
analysis of the experimentally observed T-jump relaxation rate ($k_R$) and the folding equilibrium constants ($K_{eq}$) obtained from our temperature-dependent CD measurements (because $k_R = k_F + k_U$ and $K_{eq} = k_F/k_U$) (see section 1.3.2).

### 4.2.6 Folding and unfolding kinetics

Because all our equilibrium and kinetic data fit the two-state model quantitatively, the effective folding and unfolding rates ($k_{F,eff}$ and $k_{U,eff}$) involving the crossing of the main free-energy barrier at a particular temperature can be estimated. We find that the effective folding and unfolding rates ($k_{F,eff}$ and $k_{U,eff}$) of each of the

![Figure 4.8: Representative dataset probing the T-jump relaxation dynamics of peptide (i + 4)EK in the amide I' region following a T-jump from 293 to 301 K. (a) Three-dimensional representation of the observed relaxation kinetics corrected for solvent absorption changes. The difference in absorption ($\Delta A$) is presented as function of frequency and time. (b) Transient spectra for selected delay times compared to the (scaled) FTIR difference spectrum. (c) Time course of the T-jump relaxation monitored at 1630 cm$^{-1}$ ($\alpha$-helix) and 1658 cm$^{-1}$ (random-coil). The relaxation kinetics are globally fitted to a single-exponential decay function (solid curve): $\Delta A(t) = A_0 + A_1 \exp(-t/\tau)$, with an observed time-constant of $\tau = 143 \pm 4$ ns.](image-url)
investigated peptides exhibit Eyring temperature dependence. From least-squares fits of the data to the Eyring equation we obtain the effective kinetic parameters, viz., the apparent enthalpy and entropy for the formation of the transition state ($\Delta H^\text{app}$ and $\Delta S^\text{app}$), both for the folding ($U \rightarrow F$) and unfolding ($F \rightarrow U$) transitions [29]. The results are listed in Table 4.1 (folding) and Table 4.3 (unfolding).

To specifically determine the potential role of salt bridges on the (un)folding kinetics, we first compare the effective $\alpha$-helical folding rates ($k_{F,\text{eff}}$) of the different E/R, D/R, and E/K peptides in the absence of salt bridges at acidic pH (see Fig. 4.9, left column). We observe small but measurable differences in the kinetic behavior of the investigated peptides. As described in the previous chapter (see section 3.2.3), the effective folding rates at acidic pH of the ($i, i + 3$) spaced E/R peptides are slightly faster compared to the ($i, i + 3$) spaced E/R peptides. This dichotomy probably arises from a slight sequence-position dependence of the preferred rotamer conformations (and helix-forming propensity) of Glu$^0$ and Arg$^+$ with respect to each other [58]. For the D/R analogs at acidic pH, only the effective folding rates of peptide ($i + 4$)DR could be estimated (Fig. 4.9c), because the folding equilibrium constants ($K_{eq}$) for peptides ($i + 3$)DR, ($i + 4$)RD, and ($i + 3$)RD could not be reliably determined in the absence of salt-bridge effects at acidic pH. Comparison of the effective folding rates of peptides ($i + 4$)ER and ($i + 4$)DR shows that replacement of Glu$^0$ with Asp$^0$ only marginally slows down the folding. Notably, the effective folding rates of the Lys-based peptides at acidic pH are slightly slower compared to the Arg-based variants (Fig. 4.9e). In both cases, these effects might arise from differences in the helix-forming propensity of the involved amino-acid residues [56]. In contrast to the Arg-based peptides, the Lys-containing sequence do not show any dependence of the kinetics on the position and order of Glu$^0$ and Lys$^+$ (Fig. 4.9e).

### 4.2.7 Effect of charge spacing and order

Fig. 4.9 (right column) shows the effective folding rates ($k_{F,\text{eff}}$) of the different E/R, D/R, and E/K peptides at neutral pH, and reveals that the ability to form salt bridges has a significant impact on the folding kinetics of the investigated $\alpha$-helices. We find that a salt bridge can both speed up and slow down the rate of $\alpha$-helix formation, depending on the type of charge pairs and their geometry. As we have found previously (see section 3.2.3), the rates of $\alpha$-helix folding of the E/R peptides at neutral pH show the same trend as their structural stability, viz., ($i + 4$)ER > ($i + 3$)ER $\approx$ ($i + 4$)RE > ($i + 3$)RE (see Fig. 4.9b). A similar correlation between folding kinetics and thermodynamics is found for the D/R sequence patterns, as the effective folding rates show the trend ($i + 4$)DR > ($i + 3$)DR > ($i + 4$)RD (see Fig. 4.9d). The folding rates of peptide ($i + 3$)RD could not be reliably estimated as the UV-CD measurements did not allow a quantitative determination of the folding equilibrium constant ($K_{eq}$). As in the case of the Arg-based peptides, the folding rates of the Lys-containing peptides show the same trend as the thermal stability of the folded structures, viz., ($i + 4$)EK > ($i + 3$)EK $\approx$ ($i + 4$)KE > ($i + 3$)KE (see Fig. 4.9f). Thus, the effective folding rates within each particular
Figure 4.9: Eyring plots of the effective folding rates ($k_{F, eff}$) of the different E/R peptides (top), D/R peptides (centre), and E/K peptides (bottom), measured in the absence (left) and presence (right) of salt-bridge effects. The solid lines are least-square fits to equation 1.2. For better comparison, in the plots of $k_{F, eff}$ at neutral pH (salt-bridge effects) the fits to $k_{F, eff}$ at acidic pH (no salt bridges) are shown as dotted lines for peptides $(i+4)$ER and $(i+3)$RE (top), $(i+4)$DR (centre), and $(i+4)$EK and $(i+3)$EK (bottom). The experimental values for $\Delta H_{app}^\ddagger (U \rightarrow F)$ and $\Delta S_{app}^\ddagger (U \rightarrow F)$ were obtained from fitting of the data to the equation 1.2, and are summarized in Table 4.1.
set of E/R, D/R, or E/K salt-bridge combinations show a similar sequence-position
dependence of the charged side chains. In all cases, the ability to form an AB-
oriented salt bridge spaced at \((i, i + 4)\) speeds up the rate of \(\alpha\)-helix formation
(Fig. 4.9 right column, red data points). By contrast, a BA-oriented salt bridge
spaced at \((i, i + 3)\) slows down \(\alpha\)-helical folding (Fig. 4.9 right column, purple
data points). Hence, inhibiting the formation of this unfavorable BA-oriented salt
bridge upon protonation of Glu or Asp has an accelerating effect on the folding of
the investigated \(\alpha\)-helices.

The similar tendency in kinetic behavior observed within each particular set
of sequence patterns, probably arises mainly from the intrinsically preferred side-
chain rotamers of the charged residue pairs and their capabilities to form a salt
bridge. A geometrically (rotamERICally) optimized salt bridge may speed up the
folding due to efficient relaxation of the salt-bridging side chains to their native-
state rotamERIC conformation during the folding process. In fact, different computa-
tional investigations provide evidence that the rotamERIC state of an individual
side chain can have a significant impact on the kinetic barriers for protein fold-
ing processes, and that fast ordering of side chains to their native-state rotamERIC
conformation facilitates folding [73–75].

For the unfolding rates we find exactly the opposite behavior. Fig. 4.13 (see
section 4.5.2) shows the Eyring plots of the unfolding rates in the absence of salt
bridges at acidic pH (left column) and in the presence of salt-bridge effects (right
column). We observe that the possibility of forming salt bridges significantly
affects the unfolding kinetics of the investigated \(\alpha\)-helices. Notably, the rates of
unfolding in the presence of geometrically optimized AB-oriented salt bridges at
neutral pH (Fig. 4.13 in section 4.5.2, right column) are distinctly slower compared
to their unfolding rates in the absence of salt bridges at acidic pH (Fig. 4.13 in
section 4.5.2, left column). By contrast, the presence of unfavorable BA-oriented
salt bridges is reflected in marginally faster unfolding rates. Thus, within each set
of E/R, D/R, and E/K salt bridges, the unfolding rates show a trend opposite to
that of the folding rates, indicating that the ability to form geometrically optimized
salt bridges not only speeds up folding, but can also prevent unfolding.

### 4.2.8 Effect of the type of salt bridge (E/R, D/R, or E/K)

In Fig. 4.10, we compare the effective \(\alpha\)-helical folding and unfolding rates in the
absence (acidic pH, dotted lines in Fig. 4.10) and in the presence of different types
of geometrically optimized salt bridges (neutral pH, data in Fig. 4.10). The data
indicate significant differences in the folding kinetics of peptides containing different
type of charge pairs. We find that the rates of \(\alpha\)-helix formation at neutral pH are
in the order \((i + 4)\text{ER} \gg (i + 4)\text{DR} \gg (i + 4)\text{EK}\), indicating that an optimized E/R
salt bridge is most favorable for the speed of folding and that the Arg-containing
peptides fold faster compared to the Lys-based variant (data in Fig. 4.10a). Re-
markably, the trend observed for the folding rates of the different E/R, D/R, and
E/K peptides deviates from that observed for the conformational stability, viz.,
\((i + 4)\text{ER} \gg (i + 4)\text{DR} \approx (i + 4)\text{EK}\) (see Fig. 4.3a). This suggests that the parallel
Table 4.1: Sequences and experimentally obtained values of the apparent transition state enthalpy ($\Delta H^\text{app}_{i\rightarrow f}$) and entropy ($\Delta S^\text{app}_{i\rightarrow f}$) for the folding process ($U \rightarrow F$) of the different peptides at neutral pH (salt-bridge formation) and at acidic pH (no salt-bridge effects).

<table>
<thead>
<tr>
<th>Sequence$^a$</th>
<th>Peptide$^a$</th>
<th>$\Delta H^\text{app}_{i\rightarrow f}$ (kJ mol$^{-1}$)</th>
<th>$\Delta S^\text{app}_{i\rightarrow f}$ (J mol$^{-1}$ K$^{-1}$)</th>
<th>$\Delta H^\text{app}_{i\rightarrow f}$ (kJ mol$^{-1}$)</th>
<th>$\Delta S^\text{app}_{i\rightarrow f}$ (J mol$^{-1}$ K$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-A(EAAAR)$_3$A-NH$_2$ (i+4)ER</td>
<td>16.6 ± 1.2</td>
<td>-62.0 ± 4.9</td>
<td>15.3 ± 1.7</td>
<td>-69.3 ± 5.8</td>
<td></td>
</tr>
<tr>
<td>Ac-A(AEAAAR)$_3$A-NH$_2$ (i+3)ER</td>
<td>13.1 ± 1.1</td>
<td>-80.1 ± 3.6</td>
<td>17.5 ± 3.6</td>
<td>-67.3 ± 12.3</td>
<td></td>
</tr>
<tr>
<td>Ac-A(RAAAE)$_3$A-NH$_2$ (i+4)RE</td>
<td>11.9 ± 1.9</td>
<td>-84.7 ± 6.4</td>
<td>14.1 ± 3.4</td>
<td>-75.0 ± 11.8</td>
<td></td>
</tr>
<tr>
<td>Ac-A(AARAAE)$_3$A-NH$_2$ (i+3)RE</td>
<td>5.2 ± 1.5*</td>
<td>-115.4 ± 5.1*</td>
<td>16.7 ± 3.0</td>
<td>-72.2 ± 10.3</td>
<td></td>
</tr>
<tr>
<td>Ac-A(DAAAR)$_3$A-NH$_2$ (i+4)DR</td>
<td>13.6 ± 2.4</td>
<td>-73.1 ± 2.6</td>
<td>19.1 ± 2.1*</td>
<td>-59.9 ± 7.3*</td>
<td></td>
</tr>
<tr>
<td>Ac-A(ADAAR)$_3$A-NH$_2$ (i+3)DR</td>
<td>8.6 ± 1.7*</td>
<td>-97.8±5.5*</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Ac-A(RAAAD)$_3$A-NH$_2$ (i+4)RD</td>
<td>2.9 ± 1.8*</td>
<td>-120.3 ± 6.1*</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Ac-A(AARAD)$_3$A-NH$_2$ (i+3)RD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Ac-A(EAAAK)$_3$A-NH$_2$ (i+4)EK</td>
<td>18.7 ± 0.9</td>
<td>-60.1 ± 3.1</td>
<td>9.0 ± 4.1</td>
<td>-95.9 ± 14.2</td>
<td></td>
</tr>
<tr>
<td>Ac-A(AEAAK)$_3$A-NH$_2$ (i+3)EK</td>
<td>16.1 ± 1.4</td>
<td>-73.2 ± 4.7</td>
<td>4.7 ± 4.6</td>
<td>-110.1 ± 16.0</td>
<td></td>
</tr>
<tr>
<td>Ac-A(KAAAE)$_3$A-NH$_2$ (i+4)KE</td>
<td>16.8 ± 2.4</td>
<td>-71.9 ± 8.4</td>
<td>7.2 ± 3.7</td>
<td>-101.9 ± 12.8</td>
<td></td>
</tr>
<tr>
<td>Ac-A(AKAAE)$_3$A-NH$_2$ (i+3)KE</td>
<td>9.3 ± 4.2</td>
<td>-100.0 ± 14.3</td>
<td>7.0 ± 4.4</td>
<td>-102.1 ± 15.4</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Ac = acetyl; A = alanine; E = glutamic acid; R = arginine; D = aspartic acid; K = lysine.

*These are asymptotic standard errors [76].

between thermodynamic stability and kinetic behavior as observed within each set of E/R, D/R, and E/K sequence patterns is not necessarily a general effect. The observed differences in folding rates between peptides with different type of optimized E/R, D/R, or E/K salt bridges probably mainly originate from differences in the rotameric accessibility of the charged amino acid residues that allow salt-bridging in the folded state of the peptides. Fig. 4.10b shows the intrinsically preferred rotamer conformations of peptides (i+4)ER, (i+4)DR, and (i+4)EK in which the spatial geometry of the charged side chains in an α-helical backbone conformation is such that salt-bridge formation is favored (see section 3.4.3 for details). For each of the (i+4)AB oriented Arg-containing peptides, we find three different accessible low-energy rotamers of the Glu/Asp and Arg residues in which their relative orientation is such that formation of a salt-bridge is possible. By contrast, for the Lys-based variant only one intrinsically preferred rotamer pair which favors salt-bridging of Glu and Lys in an α-helix was found. The ability to occupy multiple salt-bridging rotamer pairs in the folded state of the Arg-based α-helices provide favorable (entropic) contributions to their free energy balance, and possibly explains the ability to fold more rapidly than the Lys-based analogs (Fig. 4.10 a,b).

Comparing the folding rates of the two Arg-containing peptides (i+4)ER and (i+4)DR at neutral pH, we observe that the Glu-containing variants fold slightly faster (data in Fig. 4.10a). However, inhibiting the formation of a geometrically optimized D/R salt bridge by lowering the pH (dotted line in Fig. 4.10a) slows down folding more than in the E/R equivalent, suggesting a larger net effect of
D/R salt bridges on the folding kinetics. This might originate from the changes in helix propensity of the acidic amino acid residues due to lowering of the pH, as it is known that the change in intrinsic helix propensity upon neutralization is larger for Asp than for Glu [56]. However, another plausible explanation for the observed larger net contribution of D/R salt bridges to the speed of folding arises from differences in the probability of the accessible side-chain rotamers and their corresponding free energies. We find that the probability of the accessible salt-bridge forming D/R side-chain rotamers is larger than that of the salt-bridge forming E/R rotamer pairs, suggesting a larger net effect on the folding rates of D/R salt bridges.

The effective unfolding rates in the presence of geometrically optimized E/R, D/R, or E/K salt bridges show the trend \((i + 4)\text{DR} > (i + 4)\text{EK} > (i + 4)\text{ER}\) (Fig. 4.10c). In contrast to the tendencies within each set of sequence patterns, the rates of unfolding of the different type of optimized E/R, D/R, or E/K salt bridges do not follow exactly the opposite trend as their folding rates. In particular, the rate of folding of the Lys-based peptide deviates from this trend. This deviation might arise from differences in the intrinsic helical propensity of the interacting amino acids and their relatively larger geometric restraints when involved in a salt bridge compared to Arg-containing salt bridges. To summarize, the rate of \(\alpha\)-helix formation is fastest in the presence of geometrically optimized E/R salt bridges. By contrast, the ability to form optimized E/R salt bridges reduces the rate of the unfolding process more effectively compared to the D/R and E/K equivalents. The observed differences in the effective \(\alpha\)-helical folding and unfolding rates of peptides containing different type of charge pairs, probably mainly arises from their relative probabilities to occupy low-energy salt-bridging rotamer pairs, their

![Graphs showing Eyring plots](image-url)
corresponding free energies, and the number of low-energy side chain conformers favoring salt-bridge formation [50–55].

4.2.9 Salt bridges as nucleating sites for folding

Table 4.1 summarizes the apparent transition state enthalpy and entropy ($\Delta H_{\text{app}}^\dagger$ and $\Delta S_{\text{app}}^\dagger$) for the folding of the investigated $\alpha$-helices (see Table 4.3 for the unfolding parameters). Since the quantitative contributions of the factors that affect the free energy of the transition state of the helix-coil transition ($\Delta G^\dagger$), including solvent accessibility and viscosity [78], are presently still under debate, we will mainly focus on how the presence (neutral pH) or absence (acidic pH) of different type of salt bridges changes the apparent transition enthalpy and entropy ($\Delta H_{\text{app}}^\dagger$ and $\Delta S_{\text{app}}^\dagger$) of the folding ($U \rightarrow F$) and unfolding ($F \rightarrow U$) transitions of the investigated $\alpha$-helices. In the absence of salt bridges, the transition enthalpy and entropy of folding ($\Delta H_{\text{app}}^\dagger$ and $\Delta S_{\text{app}}^\dagger$) of different peptides within the same set of salt-bridge combinations are indistinguishable to within our experimental uncertainty (Table 4.1). Comparing the transition enthalpy and entropy of folding ($\Delta H_{\text{app}}^\dagger$ and $\Delta S_{\text{app}}^\dagger$) in the presence of different types of optimized salt bridges ($(i + 4)\text{ER}$, $(i + 4)\text{DR}$, and $(i + 4)\text{EK}$), we observe only marginal differences. However, within each set of sequence patterns we observe a dichotomy between the folding transition enthalpy and entropy ($\Delta H_{\text{app}}^\dagger (U \rightarrow F)$ and $\Delta S_{\text{app}}^\dagger (U \rightarrow F)$) of peptides with AB- and BA-oriented salt bridges at neutral pH (Table 4.1). We find that fast-folding peptides that are stabilized by the formation of AB-oriented salt bridges have significantly higher transition enthalpies of folding ($\Delta H_{\text{app}}^\dagger (U \rightarrow F)$) compared to slow-folding peptides that contain geometrically unfavorable BA-oriented salt bridges. The unfavorable contribution of the transition enthalpy ($\Delta H_{\text{app}}^\dagger (U \rightarrow F)$) to the free energy barrier of AB-oriented $\alpha$-helices implies that a substantial entropy-enthalpy compensation must be responsible for the faster folding rates in the presence of salt bridges with an optimized geometry. Examination of the kinetic parameters within each of the different sets of salt-bridge combinations in Table 4.1 indeed shows that the apparent transition-state entropies for folding $\Delta S_{\text{app}}^\dagger (U \rightarrow F)$ of peptides with AB-oriented salt bridges are significantly higher than those of the slow-folding BA-type peptides, so that the accelerating effect of geometrically optimized salt bridges on the folding kinetics has an entropic origin (a change in $\Delta S_{\text{app}}^\dagger (U \rightarrow F)$). Thus, our results indicate that geometrically optimized salt bridges have an organizing rather than an energetic effect on the kinetics of $\alpha$-helix formation.

A plausible explanation for this entropic effect could be that geometrically (rotamERICally) optimized salt bridges facilitate the nucleation of the $\alpha$-helix. The NH group of each amide residue involved in an $\alpha$-helical backbone conformation donates a hydrogen bond to the C=O group of the amino acid positioned four residues earlier. Since the formation of a single or only a few consecutive hydrogen bonds requires a large loss of entropy, a certain minimum number of helical residues is needed to initiate $\alpha$-helix formation. As a consequence, the initial step in the folding of $\alpha$-helices involves the crossing of a ‘nucleation’ free-energy barrier which
separates states with long stretches of helix from those which have only a few scattered helical residues, a phenomenon analogous to crystal nucleation [79, 80]. Our results suggest that (rotamERICally) optimized salt bridges may facilitate rapid folding by acting as a nucleation site, thereby initiating the formation of local secondary structure elements and restrict the rate-determining search through the conformational space of the entire protein or peptide. This nucleating effect may even extend beyond the backbone conformation: in globular peptides and proteins, a nucleating effect may also result from the fast relaxation to the native rotameric state of a single side chain. In fact, a computational investigation of the side-chain relaxation in the folding process of Protein G indicates that secondary-structure formation is facilitated by the fast ordering of folding nucleus [74, 75]. This fast relaxation process of side chains belonging to the folding nucleus not only requires an optimized spatial geometry of the involved residues with respect to each other, but also ordering in their native-state rotamer conformation [74, 75]. In addition, simulation studies on the Trp-cage miniprotein have shown that restraining the rotameric state of a single side chain that occupies a prominent role in the folding process to its native-state rotamer significantly lowers the kinetic barrier for folding of the entire miniprotein [73].

4.3 Possible implications for protein folding

Our results show a correlation between the thermodynamic stability of the native structure (\(\Delta G_{FU}\)) and the folding rates (\(\Delta G^\ddagger_{U \rightarrow F}\)) of different peptides within the same set of salt-bridge combinations. We find that geometrically (rotamERICally) optimized salt bridges, which stabilize the folded conformation, also enhance the ability to fold rapidly, while destabilizing salt bridges rather reduce the rate of the folding process. Comparing the relative trends of the conformational stability and kinetic behavior of salt bridges formed between different type of charge pairs we find not such a correlation, indicating that the observed correlation between thermodynamic and kinetic effects of salt bridges within each set of sequence patterns is not necessarily a general effect. This observation suggests an interesting explanation for the presence of non-stabilizing, evolutionarily conserved salt bridges that exist in many proteins. Many solvent-exposed salt bridges are known to be only marginally stabilizing [17, 25] or even destabilizing [15, 16]. The contribution of buried salt bridges to protein stability has been questioned as well, because the free-energy contribution of the salt bridge has to be sufficient to cancel both the high free-energy cost required for desolvation of the charged residues during the folding process [6] and the entropic penalty caused by the restriction of a buried salt bridge to a single rotameric state [51, 52]. On the other hand, the hydrophobic protein interior also facilitates stronger electrostatic interactions between the salt-bridging side-chains due to reduced solvent screening [9]. A classical example of a stabilizing salt bridge is the Asp–His salt bridge buried in the hydrophobic core of lysozyme that lowers the free energy of the native fold by 10–20 kJ mol\(^{-1}\) [14]. By contrast, a buried Glu–Arg salt bridge in Arc repressor of bacteriophage P22
destabilizes the native structure [20], and the formation of this salt bridge has been proposed to be the rate limiting step in the folding process [30]. Moreover, MD simulations have shown that the formation of the Asp–Arg salt bridge deeply buried in the protein core of human salivary α-amylase is highly destabilizing and increases the free energy of the folded state with as much as 44 kJ mol$^{-1}$ [81]. The question arises as to the possible evolutionary advantages of the conservation of such (highly) structure-destabilizing salt bridges in proteins functioning in the living cell.

In the present study, we find that solvent-exposed salt bridges can have a strong impact on the kinetics of folding and unfolding. In particular, geometrically optimized salt bridges accelerate folding, and slow down unfolding. Our results show that the increased folding rates caused by optimized salt bridges are an entropic effect, possibly because the salt bridge facilitates nucleation of the folding process. Such kinetic effects of salt bridges might be a more general phenomenon, in which the equilibrium function (stabilizing or destabilizing) of a salt bridge is of secondary importance. By acting as a nucleation site, a salt bridge might then stabilize the transition state and/or a certain folding intermediate, but not necessarily the final, folded state. For several proteins, there exists evidence for such a purely kinetic function of salt bridges. For example, the solvent-exposed salt bridge formed between Glu and Arg in Staphylococcal nuclease has been shown to contribute only marginally to the structural stability of the folded state (2.1 kJ mol$^{-1}$) [82]. However, simulation studies indicate that this salt bridge can create significant kinetic barriers for the unfolding process, thereby ‘locking’ the protein in its native conformation and preventing the protein from unfolding [35, 36]. Evidence for a kinetic function of salt bridges has also been observed during the early stages of ATP hydrolysis by myosin, in which a Glu–Arg salt bridge stabilizes the transition state of hydrolysis [83]. Finally, experimental evidence shows that the presence of an Arg–Asp salt bridge in triosephosphate isomerase [33] and an Arg–Glu salt bridge in mouse Paneth cell α-defensin cryptdin-4 [40], both play a critical role for efficient and correct folding of the native structures, rather than having a stabilizing impact on the native folds. The impact of salt bridges on the kinetics of α-helix formation observed here may thus be a more general phenomenon, and might explain the evolutionary pressure for the conservation of apparently non-functional (destabilizing) salt bridges in many proteins.

### 4.4 Conclusions

Our results provide insights into the effect of salt bridges on the kinetics of folding. We find significant differences in the folding and unfolding rates of α-helical peptides which differ only in the type of charge pairs (either E/R, D/R, or E/K), their relative distance and order in the peptide sequence. Geometrically (rotamerically) optimized salt bridges can accelerate α-helical folding. Interestingly, the unfolding free-energy barrier is also significantly affected by optimized salt bridges, as their presence reduces the rate of α-helical unfolding. By contrast, unfavorably
oriented salt bridges reduce the rate of folding, and marginally increase the rate of unfolding. The enthalpic contribution of the salt bridge with optimized geometry to the free energy barrier for folding is unfavorable, and the increase of the folding rate is a purely entropic effect, indicating that the salt bridges have an organizing, possibly nucleating, effect. The kinetic influence of salt bridges on folding observed here might explain the biological function of apparently non-functional (destabilizing) salt bridges in biologically active proteins.
4.5 Additional methods and data

4.5.1 Materials and methods

The peptides (consisting of the sequences as listed in Table 4.1) were purchased from GL Biochem (Shanghai). Peptide purity ($\geq 95\%$) was assessed by reversed-phase HPLC, and the peptides were lyophilized against a 35% DCl/D$_2$O solution to remove residual trifluoroacetic acid (TFA) and to achieve H/D-exchange. Stock solutions of 12–16 mM were prepared by directly dissolving lyophilized solids 50 mM KD$_2$PO$_4$/K$_2$DPO$_4$ D$_2$O buffer (pH*= 7.0 or pH*= 2.0) For measurements under neutral conditions (pH*= 7.0), the pH* of the peptide solutions was adjusted by addition of NaOD solution (the volume added was less than 1% of the peptide solution). The peptide samples used in the IR measurements were kept at 12–16 mM concentration between 2 mm thick CaF$_2$ windows separated by a 50 $\mu$m Teflon spacer. All CD experiments were performed at peptide concentrations of 40 $\mu$M in 20 mM phosphate buffer (pH= 6.8 or pH= 2.0) using 2 mm quartz cuvettes. A detailed description of the steady-state UV-CD and FTIR, and computational methods can be found in section 3.4. The transient $T$-jump IR-probe experiments were performed using the optical setup described in section 2.2.1.

4.5.2 Additional experimental data

Thermodynamic parameters obtained from the UV-CD data

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$\Delta H_{UF}$ (kJ mol$^{-1}$) pH = 7.0 (with salt bridges)</th>
<th>$T_m$ (K)</th>
<th>$\Delta H_{UF}$ (kJ mol$^{-1}$) pH = 2.5 (no salt bridges)</th>
<th>$T_m$ (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i + 4)ER</td>
<td>40.2 ± 1.6</td>
<td>312.9 ± 1.2</td>
<td>41.8 ± 2.4</td>
<td>295.7 ± 1.4</td>
</tr>
<tr>
<td>(i + 3)ER</td>
<td>41.0 ± 4.1</td>
<td>289.1 ± 1.1</td>
<td>36.5 ± 1.9</td>
<td>275.4 ± 2.5</td>
</tr>
<tr>
<td>(i + 4)RE</td>
<td>47.4 ± 1.5</td>
<td>290.0 ± 1.2</td>
<td>47.4 ± 2.5</td>
<td>294.1 ± 3.2</td>
</tr>
<tr>
<td>(i + 3)RE</td>
<td>51.9 ± 5.9</td>
<td>270.0 ± 2.3</td>
<td>36.9 ± 3.4</td>
<td>272.6 ± 0.7</td>
</tr>
<tr>
<td>(i + 4)DR</td>
<td>46.2 ± 5.5</td>
<td>294.0 ± 2.0</td>
<td>27.5 ± 5.0</td>
<td>258 ± 4.7</td>
</tr>
<tr>
<td>(i + 3)DR</td>
<td>51.0 ± 3.0</td>
<td>273.5 ± 1.7</td>
<td>62.6 ± 3.2</td>
<td>273 ± 4.1</td>
</tr>
<tr>
<td>(i + 4)RD</td>
<td>56.9 ± 2.1</td>
<td>272.0 ± 1.5</td>
<td>62.6 ± 3.2</td>
<td>272.0 ± 4.1</td>
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<tr>
<td>(i + 3)RD</td>
<td>55.1 ± 3.9</td>
<td>274.8 ± 1.5</td>
<td>65.7 ± 6.1</td>
<td>287.2 ± 1.4</td>
</tr>
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</table>

All values shown are the weighted average of at least three measurements. The corresponding errors indicate the weighted standard error of the mean from minimal three measurements.
Figure 4.11: Low-temperature UV-CD spectra of the different E/R peptides (top), D/R peptides (centre), and E/K peptides (bottom) collected at 274 K and under acidic (no salt bridges, left) and neutral (salt bridges, right) pH conditions.

Infrared spectrum as a probe of the Glu and Asp protonation state

Fig. 4.12 shows the amide I’ region of the IR spectra of each of the investigated peptides at low temperature (274 K). The spectral feature arising from the $\nu_{as}(C=O)$ mode of the COO$^-$ group of Glu$^-$ observed at 1565 cm$^{-1}$ in the FTIR spectra of Arg-based peptides at neutral pH (a, e) shifts to higher frequency upon protonation and appears at 1705 cm$^{-1}$ (the $\nu(C=O)$ mode of the Glu COOH group) [48]
in the FTIR spectra collected at acidic pH (b, f). The CO-stretching mode of the COO\textsuperscript{−} group of Asp\textsuperscript{−} arising at 1586 cm\textsuperscript{−}1 (c) shifts to 1713 cm\textsuperscript{−}1 (d) upon protonation [48]. This unambiguously demonstrates that salt-bridge formation under acidic pH conditions is hindered due to protonation of the carboxylate groups of Glu or Asp.

Figure 4.12: Normalized equilibrium FTIR spectra in the amide I\prime region obtained at 274 K of each of the different E/R peptides (top), D/R peptides (centre), and E/K peptides (bottom) measured at acidic (no salt bridges, left) and neutral (salt bridges, right) pH. The insets compare the amide I\prime centre frequency, and show the same trend in relative helicity as derived from the UV-CD spectra (see Fig. 4.11).
Eyring plots of the effective unfolding rates

Figure 4.13: Eyring plots of the effective unfolding rates ($k_{U,e}$) of the different E/R peptides (top), D/R peptides (centre), and E/K peptides (bottom) measured at acidic (no salt bridges, left) and neutral (salt bridges, right) pH. The lines are least-squares fits to equation 1.2. For better comparison, in the plots at neutral pH (right), the fits at acidic pH (no salt bridges) are shown as dotted lines for peptides ($i+4$)AB and ($i+3$)BA. The experimental values obtained for $\Delta H_{\text{app}}(F \rightarrow U)$ and $\Delta S_{\text{app}}(F \rightarrow U)$ at acidic and neutral pH are listed in Table 4.3.
Table 4.3: Experimentally obtained values for $\Delta H_{\text{app}}^\ddagger$ and $\Delta S_{\text{app}}^\ddagger$ for the unfolding (F $\rightarrow$ U) transitions of the four investigated peptides obtained at neutral pH and at acidic pH.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>pH = 7.0 (with salt bridges)</th>
<th>pH = 2.5 (no salt bridges)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta H_{\text{app}}^\ddagger$ (F $\rightarrow$ U) (kJ mol$^{-1}$)</td>
<td>$\Delta S_{\text{app}}^\ddagger$ (F $\rightarrow$ U) (J mol$^{-1}$ K$^{-1}$)</td>
</tr>
<tr>
<td>(i+4)ER</td>
<td>56.3 ± 1.7</td>
<td>64.9 ± 5.4</td>
</tr>
<tr>
<td>(i+3)ER</td>
<td>62.4 ± 0.8</td>
<td>87.7 ± 2.7</td>
</tr>
<tr>
<td>(i+4)RE</td>
<td>60.8 ± 1.3</td>
<td>84.0 ± 4.3</td>
</tr>
<tr>
<td>(i+3)RE</td>
<td>57.8 ± 0.8</td>
<td>78.2 ± 2.6</td>
</tr>
<tr>
<td>(i+4)DR</td>
<td>57.7 ± 1.8</td>
<td>76.8 ± 5.9</td>
</tr>
<tr>
<td>(i+3)DR</td>
<td>58.1 ± 1.3</td>
<td>83.9 ± 4.3</td>
</tr>
<tr>
<td>(i+4)RD</td>
<td>58.4 ± 11.0</td>
<td>84.3 ± 3.7</td>
</tr>
<tr>
<td>(i+3)RD</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>(i+4)EK</td>
<td>64.2 ± 0.7</td>
<td>94.8 ± 2.2</td>
</tr>
<tr>
<td>(i+3)EK</td>
<td>61.7 ± 0.7</td>
<td>89.1 ± 2.5</td>
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<tr>
<td>(i+4)KE</td>
<td>69.3 ± 3.0</td>
<td>112.0 ± 3.0</td>
</tr>
<tr>
<td>(i+3)KE</td>
<td>63.5 ± 1.1</td>
<td>97.6 ± 3.7</td>
</tr>
</tbody>
</table>

4.5.3 Additional MD results

Figure 4.14: Helical hydrogen bond (hhb) time traces for each of the peptides.
References


Optimized E/R, D/R, and E/K salt bridges speed up the rate of α-helix formation


