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Comparative studies on the dynamics of crosslinked insulin

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Abstract. Molecular dynamics simulations were carried out on an insulin crosslinked between the N-terminal A chain and the C-terminal B chain to form a so-called mini-proinsulin: N^{α}-^{A1}-N^{ε}-^{B29}-diaminosuberoyl insulin (DASI). To investigate the influence of crosslinking on the dynamics of the insulin moiety, the bridge was removed from a transient DASI structure and simulation was carried on independently with the then unlinked (ULKI) as well as with the crosslinked species. The effects of crystal packing and quaternary interactions were checked by simulating both types of monomers and dimers known from the hexamer structure. All simulations were compared to previous ones of native insulin. DASI shows general similarity to the native simulations in most parts of the structure. Deviations are visible in the segments to which the bridge is directly connected, i.e. their flexibility is reduced. Upon removal of the bridge the ULKI simulations reapproach those of native insulin. The influence of the bridge spreads over the whole molecule, but all of its main structural features remain intact. The simulations suggest that the displacement of the C-terminal B chain of native insulin, considered important for receptor interaction, is prevented by the bridge, which also partially shields some binding residues. This is in accordance with the poor biological potency of A1-B29-crosslinked insulins.

Key words: Molecular dynamics simulation – Insulin – Crosslinked insulin – Single chain insulin – Active conformation

Introduction

Nearly all of the hundreds of insulin derivatives or analogues prepared during the past 25 years turned out to be functional agonists, sometimes despite significant perturbation of the native structure. Only very few cases are known where a dramatic loss of receptor binding capacity and biological activity is associated with a perfectly preserved three-dimensional structure. Most revealing examples are insulins crosslinked between the A chain N-terminus and the C-terminus of the B chain and single chain insulin. Bifunctional reagents for crosslinking were introduced into peptide chemistry by Zahn (1950). Using them as a "molecular yardstick" it was shown that the amino functions of A1 and B29 are in close proximity (Zahn and Meienhofer 1957) more than a decade before the X-ray structure was solved (Adams et al. 1969). Preparation of A1-B29 crosslinked insulins for structure/function studies was started by Brandenburg et al. (Brandenburg et al. 1971, 1972; Brandenburg and Wollmer 1973; Brandenburg et al. 1973a, b; Lindsay 1971) and taken up again recently by Tager and coworkers (Nakagawa and Tager 1989; Brems et al. 1991). The bridge – though a short unbiological substitute of the C-peptide of proinsulin – mediates correct folding and correct cysteine pairing in high yield. Hence, A1-B-29 crosslinked insulin was apostrophized "mini-proinsulin" (Brandenburg and Wollmer 1973). The bridge is easily accommodated, is perfectly compatible with dimer and hexamer formation (Dodson et al. 1980; Cutfield et al. 1981) and is assumed not to cover surface residues recognized to be important for receptor interaction (Pullen et al. 1976; Dodson et al. 1983). Yet on the other hand it drastically impairs receptor binding and biological activity, the shorter the bridge the greater the impairment (see Fig. 1) (Brems et al. 1991). Single chain insulin with A1 directly peptide linked to the carboxylate group of B29 is the only insulin known to be completely inactive (Markussen et al. 1985; Derewenda et al. 1991).

X-ray analyses of single chain and $N^{\alpha-A1}$-$N^{\varepsilon-B29}$-diaminosuberoyl insulin (DASI) have confirmed that the

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Abbreviations: DASI-insulin (DASI), bovine N^{α}-^{A1}-N^{ε}-^{B29}-diaminosuberoyl insulin; ULK-insulin (ULKI), Native beef insulin with the bridge of DASI removed

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native structure is nearly unperturbed. The study reported here is on the latter, whose bridge closely matches the separation of its heads in the native structure. The only explanation for its 7% activity that remains is that the bridge interferes with the hormone's inherent flexibility. Flexibility seems to be required for insulin to adopt the "active" conformation (Cutfield et al. 1981; Hua et al. 1992 b, 1993 c; Nakagawa and Tager 1993), somewhat different from those in the several X-ray structures known. The full or even enhanced activity of des[B26-B30]pentapeptide-insulin-B25-amides suggests that, prior to or upon binding to the receptor, the C-terminal B chain has to be displaced. Thereby underlying residues are uncovered which seem to participate in receptor contact. It is obvious that an A1-B29 bridge must interfere with this movement or even prevent it. Increasing length will allow for more flexibility and, hence, for more activity until excessively long crosslinks (beyond the maximum in Fig. 1) presumably interfere with receptor binding mainly by steric hindrance. A NMR study on human proinsulin (Weiss et al. 1990) has, however, revealed shifts in aromatic resonances and characteristic NOEs of the hydrophobic core resulting from a perturbation by the connecting peptide. D/H exchange and denaturation studies were carried out to assess the flexibility and stability, respectively, of A1-B29 crosslinked insulin experimentally (Brems et al. 1991).

We have studied the influence of the A1-B29 DASI bridge on the simulated dynamics of the molecule. The results of previous molecular dynamics (MD) simulations of native insulin are in general agreement with X-ray, NMR and neutron scattering data (Krüger et al. 1987; Caves et al. 1991; Mark et al. 1991, Kline and Justice 1990; Hua et al. 1991; Knetgel et al. 1991; Jorgensen et al. 1992; Wlodawer et al. 1989; Wroblowski 1993). This computational technique allows one to explore the conformational space normally accessible for the molecule. Details of the dynamics are obtained on the atomic level, whereas the effects observed in D/H exchange or denaturation experiments, for instance, could not be localized. The experimental difficulties encountered in 2D-NMR-studies on proinsulin and single chain insulin have also left room for different interpretations. MD simulations thus prove to be a valuable source of information, in complementing the limiting results of experimental techniques as well as in their own right. The X-ray coordinates of DASI served as the starting geometry. Simulations were performed with molecules 1 and 2 of the asymmetric unit cell and with two types of dimers to see the influence of the surrounding crystal field. They were compared with one another and with previous simulations of native insulin. An "experiment" in which the crosslink was released during the course of the simulation proved particularly confirmative.

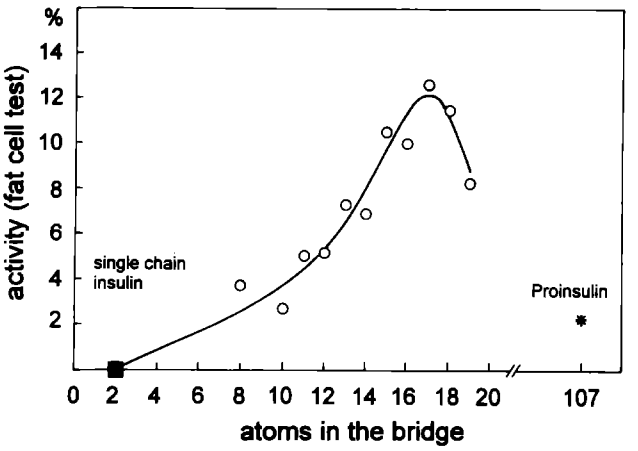

Fig. 1. The activity of A1–B29 crosslinked insulins in the isolated fat cell assay as a function of the length of the bridge. ○ crosslinked between $N^{\alpha-A1}$ and $N^{\varepsilon-B29}$ (atoms in the bridge include $C^{\beta-\varepsilon-B29}$ and $N^{\varepsilon-B29}$). ■ single chain insulin crosslinked between $C^{\alpha-A1}$ and $C^{\alpha-B29}$ by a main chain peptide bond. * human proinsulin (110 atoms)
See also: Brandenburg et al. 1972; Freychet et al. 1874; Jones et al. 1976; Gliemann and Gammeltoft 1974; Kobayashi et al. 1989

Computational procedure

The molecular dynamics simulations were performed with the program package GROMOS. All force field parameters were taken from the GROMOS 87 program manual (van Gunsteren and Berendsen 1987). They were identical with the ones used in a previous simulation of native insulin (Krüger et al. 1987). Vacuum conditions were chosen with the "discharged" parameter set for partial charges. Initial velocities were taken from a Maxwellian distribution and coupled to a heat bath with a temperature of 300 K. The bond lengths were kept constant with the SHAKE algorithm and the integration step was set equal to 2 fs. Cut-off radii $R_{c1} = 7.0$ Å and $R_{c2} = 20.0$ Å were used for the Lennard-Jones and for the Coulomb interactions, respectively. The structures were energy minimized before starting the simulation using the steepest descent procedure. All simulations except the ULKI one were carried over a period of 120 ps. The simulations of the monomer molecules were prolonged to 500 ps to check that the system does not drift further away from its initial structure. During the first 60 ps the system was allowed to equilibrate and the analysis period was from ps 60 to 120. The ULKI simulation, i.e. the one with "unlinked" DASI, was started with the structures of the 100th ps. The analysis period for this simulation and the corresponding reference simulation was from ps 110 to 150. Independent simulations of molecules 1, 2 and the dimer of native insulin in solution were used for comparison. The initial structures were identical to the ones used in the native vacuum simulation. Periodic boundary conditions were applied including 1656, 1672, 1653 water molecules, respectively, and 100 ps were simulated for each molecule at 300 K. (For further details see Wroblowski 1993). The analysis of the trajectories was performed using the programs GROMOS, ACAMOD (Krüger et al. 1988) and SIMLYS (Krüger et al. 1991; Krüger and Szameit 1992) and model building with CHEM-X (Program CHEM-X, developed and distributed by Chemical Design Ltd., Oxford, UK).

Although we have performed extensive simulations on insulin under solvent conditions, vacuum conditions were

chosen in this case for the following reasons:

- Simulations of native insulin under vacuum and under solvent conditions have shown general agreement (Krüger et al. 1987; Caves et al. 1991; Mark et al. 1991; Wroblowski 1993).
- Vacuum simulations are acceptable for comparative studies.
- Comparison is intended with previous simulations of native insulin also executed under vacuum conditions.
- Under vacuum conditions the conformational space explored is wider because the damping effect of the solvent is absent.
- The structure of both DASI and ULKI are not very highly resolved and had to be slightly modified locally by model building. Therefore elaborate simulations seemed not to be indicated.

While previous simulations of native insulin (Krüger et al. 1987) were based on the porcine hormone (Baker et al. 1988), X-ray coordinates of DASI were available only for the bovine species. The X-ray structure of DASI at 3.2 Å resolution (Dodson et al. 1980; Cutfield et al. 1981) was used after a few minor modifications (see below). The sequences differ in two residues, A8 Thr →Ala and A10 Ile → Val. The crystal structures of both DASI and native insulin contain two monomers in the asymmetric unit (see Fig. 2). The molecules differ mainly in the position of the A_N-helix (due to a 40° difference in the phi angle of A6) and in the orientation of the Phe B25 residue (Dodson et al. 1979; Baker et al. 1988). In molecule 1 the side chain of Phe B25 is oriented towards its own molecule, while in molecule 2 it covers the β pleated sheet in the direction of the other protomer. The Chinese nomenclature is used to designate structure 1 and 2 (Baker et al. 1988).

The X-ray structure of bovine DASI and native pig insulin are indeed highly similar, as shown in Fig. 2 (Dodson et al. 1990; Cutfield et al. 1981). Owing to the limited resolution of the electron density, some uncertainty remains concerning the exact position of the crosslink. Lys B29 is reoriented by a rotation about the Cα peptide bond and improves the pseudo-twofold axis. The distance between $N^{\alpha\text{-}A1}$ and $N^{\varepsilon\text{-}B29}$, 13.5 and 7.5 Å in molecule 1 and 2, respectively, in the native structure is 8.2 and 7.0 Å in the DASI structure.

Calculation of the potential energy of DASI showed that one molecule has significantly higher energy than the corresponding native molecule. The high potential energy is concentrated where the bridge is attached. The disorder seen in the X-ray structure made it necessary to adjust some atoms to plausible positions. The position of the C and O in the peptide connection between A1 and the bridge (molecule 1) had to be corrected. At the side of B29 in molecule 2 a cis peptide bond was noticed. This was not corrected because the potential energy was not increased. The cis isomerism may be allowed for by the flexibility of the carbon chain.

To check the influence of crystal packing and/or quaternary structure on the dynamics of the molecule, simulations were performed with the two types of monomers and dimers shown in Fig. 3. The dimers are defined by

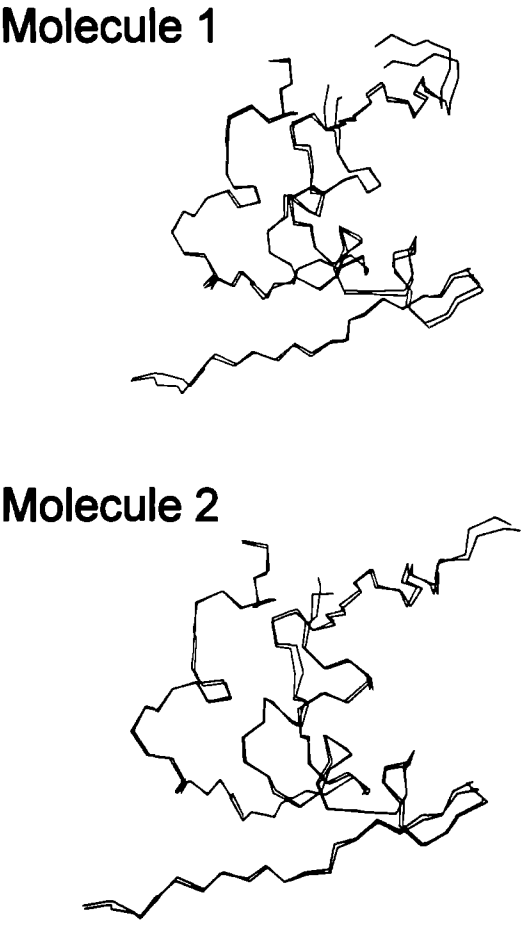

Fig. 2. Backbones of the DASI and native pig insulin, fitted to optimum superposition. Native (———); DASI (——)

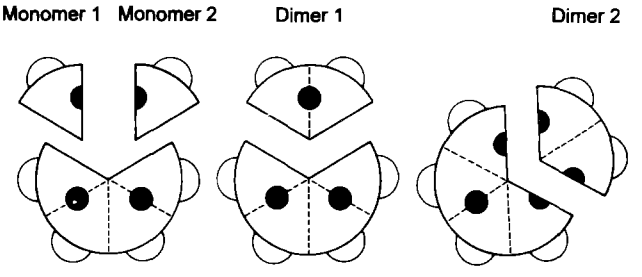

Fig. 3. Schematic representation of different nonomer and dimer forms of insulin

their alternative contact areas between monomers, according to the hexamer structure. Dimer 1 holds together through the so-called monomer/monomer contact and is the one preferentially formed in solution (Baker et al. 1988), whereas dimer 2 is held together by the so-called dimer/dimer contact.

In the course of the DASI simulation the bridge was removed from the molecule because it was expected that this would reestablish the native state. Hereby the reversibility of effects induced by the bridge can be studied. A simple comparison to the native simulation is not suffi-

cient because of the differences in sequence (beef versus pig), in structure and in resolution.

As starting structure for the ULKI simulation the transient structures of molecules 1 and 2 at ps 100 were taken. The carbonyl C atoms of the bridge were resubstituted by hydrogens and subsequently the entire structure was energy minimized. For the A1 and B29 amino nitrogens the velocities were taken from the DASI simulation. By these means it was possible to reestablish a "quasi native" state with minimal perturbations. Parallel simulations with and without the bridge were continued over the same time period up to 150 ps. Simulation time was restricted to the rather short period of 150 ps in order to maintain comparability with our simulations published earlier.

Results and discussion

The potential energy

The monomer and dimer simulations of DASI were started with energy minimized structures. After an initial drop during the first the 5 ps the potential energy remains constant at a level of about −1300 kJ per mol monomer (not shown). This is roughly 100 kJ/mol higher than determined for the equilibrated state of native insulin in our previous simulation (Krüger et al. 1987). After removal of the bridge, i.e. in the ULKI simulation, the potential energy remains at the same level. This suggests that the bridge does not cause significant strain.

The rms-positional differences

A comparative approach was chosen to distinguish the various influences. A number of simulations, representing quite a substantial data bank, was analyzed. The simulations differ in the following aspects:

- in the chemical modification through the bifunctional reagent (DASI)
- in the amino acid sequence of beef and pig insulin, different in two residues A8 Thr →Ala and A10 Ile → Val
- in the resolution of the initial X-ray structures, being 3.2 Å in DASI and 1.6 Å in native pig insulin
- in the two different molecules 1 and 2
- in the aggregation states (monomers, dimer 1 and dimer 2)
- in the modification connected with the removal of the bridge.

These structures represent a data base of insulin simulations in which different parts of the conformational space are explored. The risk that the structures are trapped in a local minimum exists even when the simulations are long (van Gunsteren and Mark 1992). This risk can be reduced if the conformational space is sampled in several independent simulations under slightly different conditions, such as initial velocities, starting geometries or surroundings. A key question is whether there still are similarities in all simulations. If so, these would be the dynamic aspects specific for insulin.

Table 1 shows an overall comparison of the rms-positional differences between the initial and all simulated structures. The values are given for the X-ray structures, the two native monomers, the two DASI monomers, the two DASI dimers and the two ULKI monomers. In all cases the simulated molecule 1 is closer to its initial X-ray structure than molecule 2. In the dimer simulations molecule 1 also remains close to its initial structure. This is in accordance with recent results of simulations under solvent conditions (see Table 2). Large deviations are however observed for molecule 2 in the simulation of dimer 2.

Table 1. Rms-positional differences between the X-ray and the averaged simulated structures after optimum fit on all Cα atoms. Averages were taken from all transient structures over 60 to 120 ps of the molecular dynamics simulations. Deviations are expressed in Å

								Dimer 1		Dimer 2			
	X1N	XC1	XC2	MDC1	MDC2	MDU1	MDU2	MDC1	MDC2	MDC1	MDC2	MD1	MD2
X1N		0.40	1.09	1.25	1.99	1.41	1.53					1.56	1.43
X2N	1.18	1.16	0.27	1.46	2.11	1.60	1.91					1.89	1.60
XC1			1.09	1.17	2.00	1.41	1.59	1.37	1.26	1.34	2.28	1.62	1.47
XC2				1.44	2.10	1.57	1.87	1.59	1.45	1.36	2.18	1.82	1.57
MDC1					1.67	0.62	1.53	1.19	1.09	1.28	2.15	1.47	1.42
MDC2						1.26	0.88	0.97	1.36	1.43	1.37	0.96	1.14
MDU1							1.26	0.90	1.03	1.10	1.82	1.12	1.16
MDU2								0.94	1.20	1.17	1.46	0.98	0.78
MDC1									1.09	0.89	1.71		
MDC2										1.38	1.76		
MDC1											1.86		
MD2												1.01	

X1N – X-ray structure native insulin of molecule 1
X2N – X-ray structure native insulin of molecule 2
XC1 – X-ray structure crosslinked insulin of molecule 1
XC2 – X-ray structure crosslinked insulin of molecule 2
MD1 – Averaged MD structure of native insulin molecule 1
MD2 – Averaged MD structure of native insulin molecule 2
MDC1 – Averaged MD structure of DASI molecule 1
MDC2 – Averaged MD structure of DASI molecule 2
MDU1 – Averaged MD structure of ULKI molecule 1
MDU2 – Averaged MD structure of ULKI molecule 2

Table 2. Rms-positional differences between the X-ray and the averaged simulated structures after optimum fit on all Cα atoms. Averages for the solution simulations were taken over all transient structures between 20 to 100 ps of the molecular dynamics simulations. All other simulations are similar to the ones in Table 1. Deviations are expressed in Å.

	Dimer 1							
	MDS1	MDS2	MDS1	MDS2	MDC1	MDC2	MDU1	MDU2
X1N	0.84	1.74	0.71	1.36				
X2N	1.67	1.60	1.43	0.92				
MDS1		1.34	0.73	1.16	1.18	1.81	1.22	1.51
MDS2			1.61	1.52	1.72	1.72	1.57	1.73
MDS1 Dimer 1				1.45				

MDS1 – Averaged MD structure of native insulin molecule 1 in solution
MDS2 – Averaged MD structure of native insulin molecule 2 in solution
X1N, N2N, MDC1, MDC2, MDU1 and MDU2 see Table 1

The smallest deviations between molecule 1 and 2 were found in the dimer 1 simulation. The monomer/monomer association thus has a twofold effect: reduction of the drift away from the X-ray structure and of the differences between monomers. In dimer 2 the deviations are much larger, which shows that the association surface formed by two dimers has no specific stabilizing effect. The ULKI simulations after the cleavage of the bridge are again very close to the simulations of the DASI monomers and dimer 1. Deviations between the ULKI simulations and dimer 2 are more pronounced but not as much as the deviation of dimer 2 from its initial X-ray structure.

Figure 4 gives some details of Table 1 in a more instructive representation. While the native simulation shows a slight convergence for molecules 1 and 2, the DASI simulation exhibits a divergence. After cleavage of the bridge in the ULKI simulation, the averaged structures converge again and occupy positions more similar to each other, separated by only 1.26 Å (see Table 1).

There is remarkable correspondence between observations made within the set of vacuum simulations of crosslinked insulin and observations within a set of simulations that had been performed before in solution, but with native insulin (Wroblowski 1993; see Table 2). In both cases molecule 1, for instance, is closer to its initial structure than molecule 2. In both cases structure 2 is stabilized when part of the classical dimer 1. This seems to exclude major vacuum effects. It may, therefore, be justified to point out, that the positional differences in Table 2 between unlinked (in vacuum) and native (in solution) are smaller than between crosslinked (in vacuum) and native (in solution) (as expected).

A more specific insight can be obtained by looking at the positional differences of secondary structure segments between the averaged simulated and their initial structure. The insulin main chains are divided into segments according to Wodak et al. (1984), which have also been used in the previous simulation of native insulin. The segments of the A chain are: the A_N-helix, residues ranging from A1 to A9, the extended strand, residues A10 to A14, and the A_C-helix, residues A12 to A19. Those of the B chain are: the extended region, residues B1 to B8, the central α-helix, residues B9 to B19, the turn including residues B20 to B24 and the β-strand, residues B22 to B29. The bridge in DASI was treated as an independent segment.

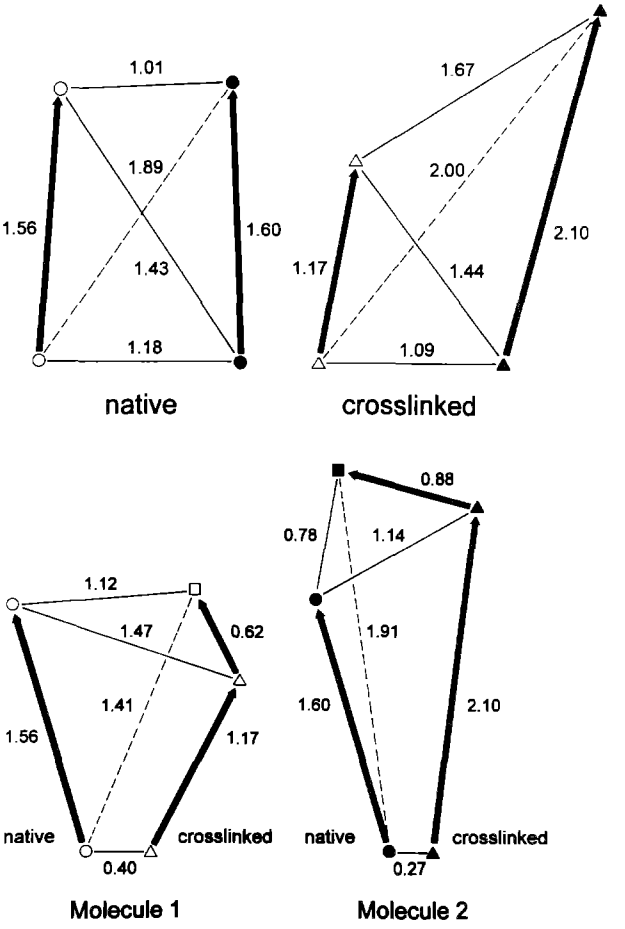

Fig. 4. Rms-positional differences (Å) between the averaged simulated structures of DASI, ULKI and native insulin and their respective X-ray structures.
○ native molecule 1 ● native molecule 2
△ crosslinked molecule 1 ▲ crosslinked molecule 2
□ unlinked molecule 1 ■ unlinked molecule 2
The broken lines with differences are not true to scale

The positional differences of these structure segments are depicted in Fig. 5. Evidently there is a similar pattern of higher or lower positional differences for a number of structure segments. One of the most stable segments is the B-helix, whereas the bridge nearly always exhibits the largest deviations. There is more divergence in molecule 2 than in molecule 1. Somewhat larger positional differ-

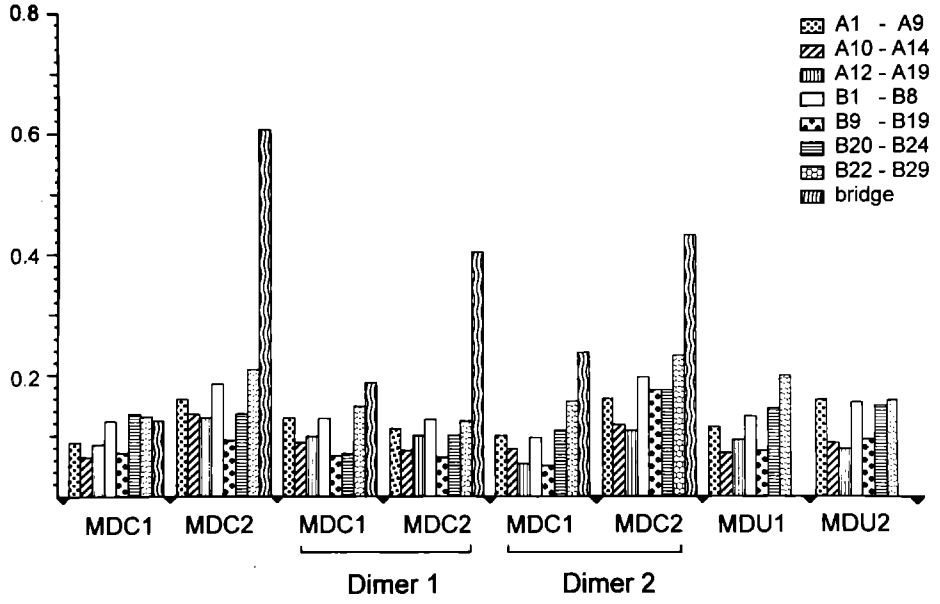

Fig. 5. Averaged positional differences of the Cα atoms between the mean simulated structures and their initial structures. Depicted are structure segments defined according to Wodak et al. (1984). The values are expressed in Å (Abbreviations see Table 1)

ences are also detectable in the A_N-helix as well as in the N- and the C-terminus of the B chain. For the A_N-helix they are a bit more elevated in molecules 2 except in dimer 1. The overall impression is that dimer 1 allows fewer deviations in nearly all segments, even in the bridge. Similar tendencies in the positional differences of the segments have also been seen in the simulation of native insulin.

In the DASI simulation the deviations of the bridge in molecule 1 and 2 are not the same. The initial coordinates have more influence than the aggregation state. The shift of the crosslink is also an indication of its mobility. The dynamics of the crosslink are influenced by the initial coordinates and the dynamics can, in turn, influence the rest of the molecule. Differences in the mobility of the crosslink do not have a dominant influence on the pattern of the positional differences among the structure segments. The same results are obtained even if the bridge is removed. Neither the aggregation state nor the bridging changes this general pattern.

Time series of the deviations

The time series of DASI molecules 1 and 2 are analyzed in more detail and compared to the simulation of native insulin. In Fig. 6 the rms-positional differences of the segments A1–A9, A10–A14, A12–A19, B1–B8, B9–B19, B22–B29 are depicted. The values are given for the DASI and native simulation at every ps. In molecule 1 of native insulin the A_N-helix shows an initial drift and remains more or less constant during the simulation. In both DASI molecules the initial deviations are smaller. The drift is less steep and the fluctuations are reduced. The A_N-helix has the highest deviations of all A chain segments in all simulations. Similarities are also observable in the other two A chain segments, the turn region A10–A14 and A_C-helix A12–A19. They have a similar level of rms-positional differences. In the B chain the helical region B9–B19 consistently shows the lowest deviations, while those in both the N and the C-terminal B chain are relatively large.

Table 3. H-bonds of the main-chain. Criteria: H-O distance ≤ 2.5 Å; N-H-O angle $\geq 135°$. Figures indicate how often an H-bond was found to exist at every full ps between the 60th 120 th ps of the simulation (in %)

H-bond	Molecule 1		Molecule 2	
	DASI	native	DASI	native
A chain:				
5 Glu – 1 Gly	95	85	99	75
6 Cys – 2 Ile	65	97	97	91
7 Cys – 3 Val	88	80	31	91
8 Ala – 4 Glu	–	10	63	2
8 Ala – 5 Gln	–	31	20	5
9 Ser – 4 Glu	2	4	5	48
9 Ser – 5 Gln	–	55	66	12
16 Leu – 12 Ser	82	77	56	80
17 Glu – 13 Leu	97	90	86	97
18 Asn – 15 Glu	2	44	–	53
18 Asn – 14 Tyr	85	–	100	–
20 Cys – 17 Glu	57	18	71	16
B-chain:				
11 Leu – 7 Cys	66	66	37	40
11 Leu – 8 Gly	7	4	20	14
12 Val – 8 Gly	86	94	80	92
13 Val – 9 Ser	68	79	90	89
14 Ala – 10 His	95	99	93	95
15 Leu – 11 Leu	99	98	90	97
16 Tyr – 12 Val	87	95	91	90
17 Leu – 13 Glu	92	93	93	87
18 Val – 14 Ala	96	91	87	97

The time series of the positional differences in the single segments of DASI indicate a number of similarities to those in native insulin. The bridge mainly affects the elements which it is bonded to, i.e. the A_N-helix and C-terminus of the B chain.

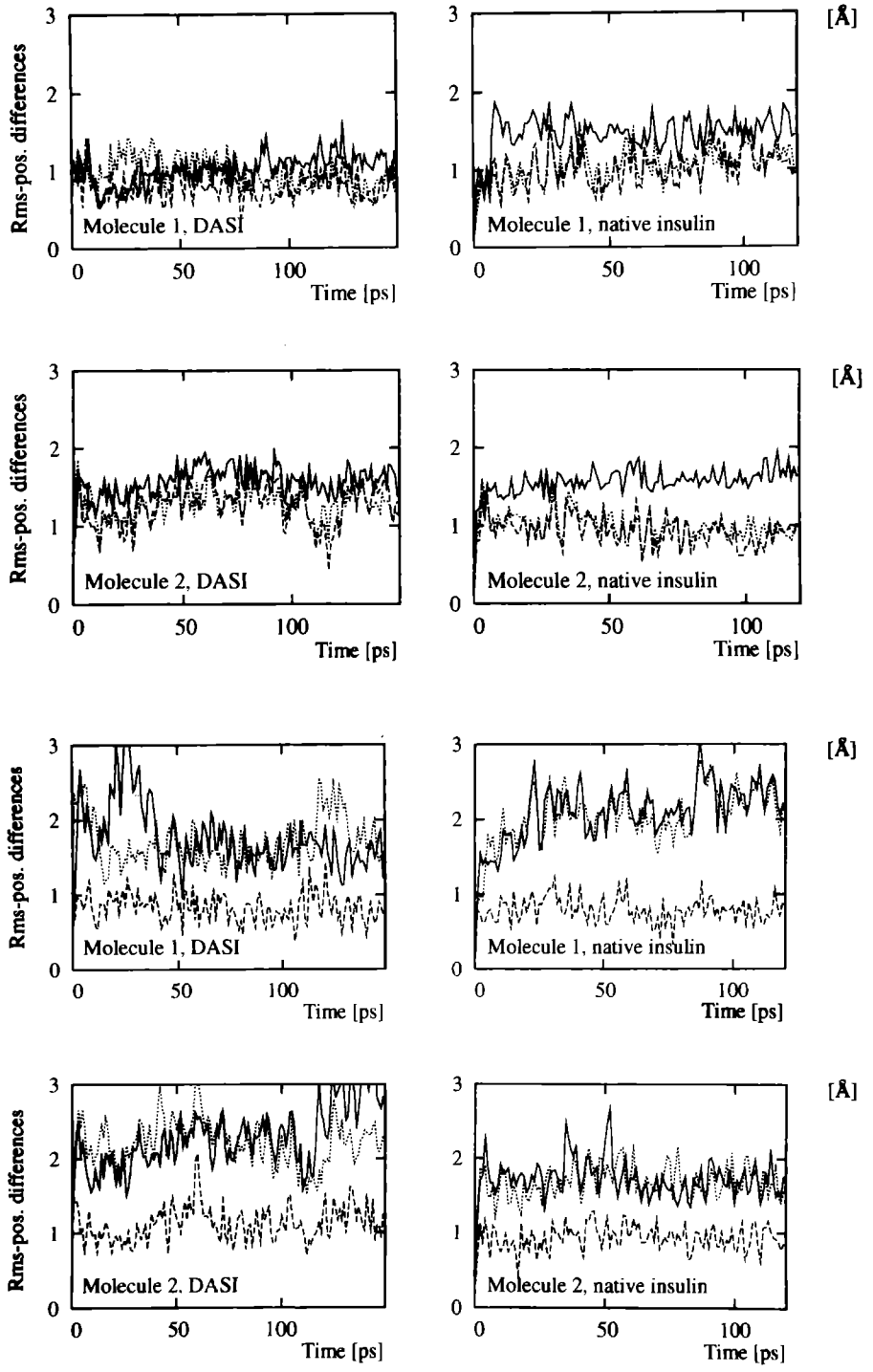

Fig. 6. Rms-positional differences between the Cα atoms of the X-ray structures and the simulated structures as a function of simulation time. *Top*: A chain: Averages over the segments A1–A10 (——); A10–A14 (– – –); A12–A19 (.....). *Bottom*: B chain: Averages over the segments B1–B8 (——); B9–B19 (– – –); B22–B29 (.....)

The H-bonds

In order to illustrate the stability of the structural segments, the H-bond patterns of the DASI and native simulation were compared. In Table 3 the lifetimes of the H-bonds are given in percentages of their existence. The A_N-helix maintains its initial heterogeneity as a mixture of π, α and 3_{10}-helix. The H-bonds between Gly A1 and Glu A5 and Ile A2 and Cys A6 have an α-helical character, but in the same region Val A3 – Cys A6 and Glu A5 – Ala A8 show a tendency towards a 3_{10}-helix. The C-terminal part of the A_N-helix is very unstable, particularly in molecule 1.

The A_C-helix has 4 H-bonds, three of α and two of 3_{10} helical character. The bond between A18 and A15 is just about vanishing in both molecules. In DASI it is replaced by a bond between A18 and A14 and in addition there is a more stable H-bond between A20 and A17. The A_C-helix is more α-helical than the A_N-helix, but turns into a 3_{10}-type at the C-terminus.

The B-helix is very stable with most of its H-bonds having lifetimes of nearly 90%. The only exception is a

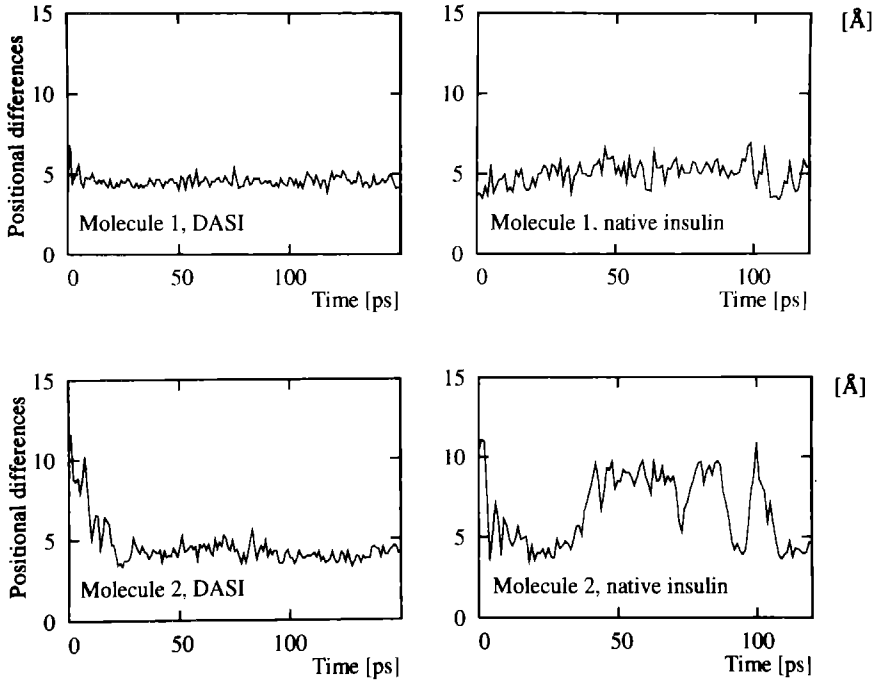

Fig. 7. The distance between atoms Nα of Gly A1 and the carboxylate C of Ala B30 as a function of simulation time

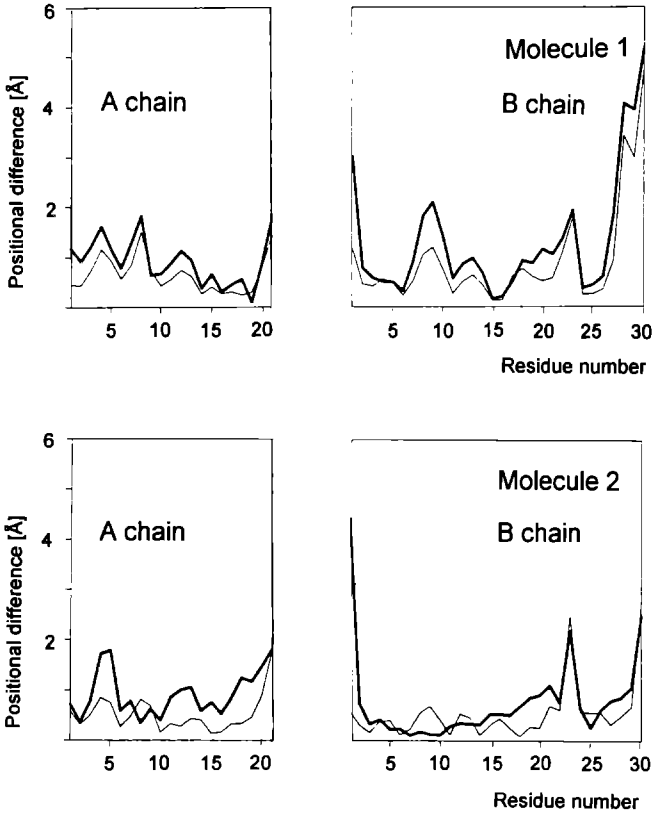

Fig. 8. Rms-positional differences between the Cα atoms of the X-ray structure of native insulin and the averaged simulated structures of DASI (——), and ULKI (—) respectively

i → i–3 bond of Leu B11. There is practically no difference between the native and DASI simulations.

A characteristic main chain H-bond exists between the imino proton of B25 and the carbonyl oxygen of A19. This H-bond is thought to play a role in the mobility of the C-terminal B chain. In DASI the bond does exist in molecule 1 and 2 with lifetimes of 95% and 88%, respectively, while for native insulin 79% and 88% are calculated. The higher values for DASI may well be due to restricted mobility of the C-terminal B chain.

In concert with the rms-positional differences and time series of the structural segments, the analysis of the H-bonds reveals various identical features in DASI and native insulin. The main differences are visible in the A_N-helix, which is also in agreement with the positional differences of this particular segment. These findings are in accordance with recent results obtained by acid-quenched amide proton exchange (Hua et al. 1993b).

The A1-B30 distance

An indicator for the mobility of the C-terminal B chain is the distance between the residues A1 and B30 (see Fig. 7). For the simulation of native insulin it has been shown that the distance differs in molecule 1 and 2. In the native molecule 1 the initial distance of about 4 Å is maintained. The same is true for molecule 1 of DASI with the motion of the termini roughly at the same level but the fluctuations reduced. For molecule 2 fluctuations between 4 and 10 Å are observed. At the beginning of the simulation the distance is similar to the highest value of the corresponding native simulation. After a few picoseconds, however, it levels off and remains constant at about 4 Å. The bridge thus restricts the mobility of the termini.

Table 4. Rms-fluctuations of the DASI, ULKI and native simulation given in Å

Simulation	A1–B25[a]	B26–B30[b]	total
MD1	0.548	0.939	0.586
MD2	0.544	0.962	0.585
MDC1	0.650	0.829	0.668
MDC2	0.660	0.684	0.663
MDU1	0.590	1.137	0.644
MDU2	0.592	1.023	0.635

[a] Cα atoms of the main-chain from residue A1 to B25
[b] Cα atoms of the main-chain from residue B26 to B30

The positional differences after release of the bridge

To demonstrate the consequences of removing the bridge from DASI the rms-positional differences between the Cα atoms of the averaged simulated structures of DASI and ULKI, respectively, and native pig insulin were calculated. It can be gathered from Fig. 8 that the ULKI simulation leads to new positions of molecules 1 and 2 in conformational space. The positional differences from the native structure are consistently smaller for ULKI than for DASI. There is a general convergence towards the native structure in both molecule 1 and 2. The presence of the bridge, as well as its removal thus affect the entire main chain. The potential energy calculated for the simulation before and after the removal of the bridge was indistinguishable from that of crosslinked insulin. It is, therefore, unlikely that the drifts were due to 'wrong' energies.

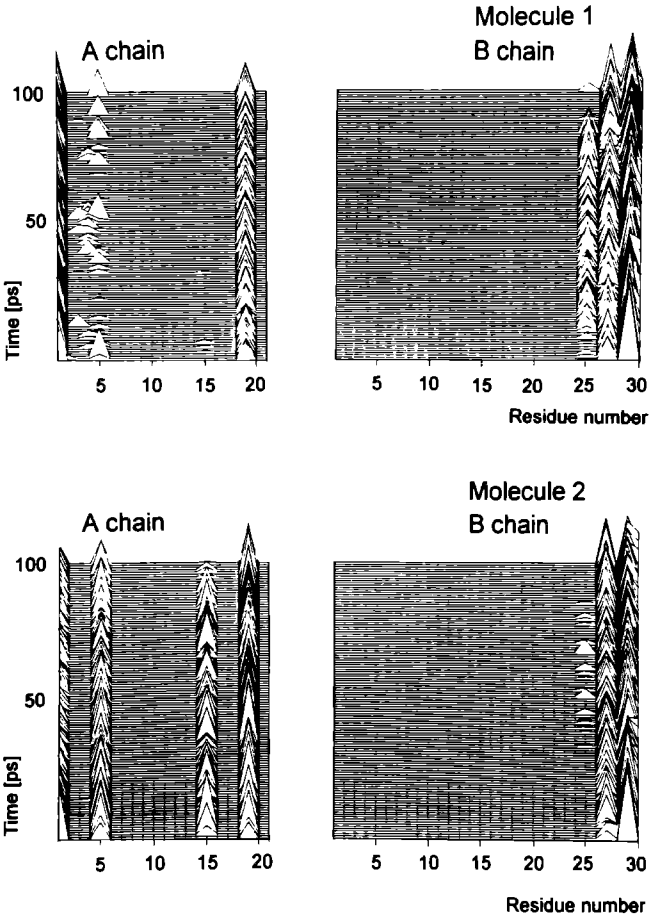

Fig. 9. Difference in solvent accessible surface for the transient simulated structures of DASI in the presence and absence of the bridge. Peaks are indicating restricted accessibility

The rms-fluctuations

The rms-fluctuations were calculated for the simulation of DASI, ULKI and native insulin (see Table 4). The bridge in DASI restricts the mobility of the B chain from B26–B30 in both molecules 1 and 2 as compared to the native simulation. This can also be seen in the restricted changes between the A chain N-terminus and the B chain C-terminus in molecule 2 (see Fig. 7). This effect seems to be partially compensated for by enhanced fluctuations everywhere else in the molecule. Fluctuations in the C-terminal B chain increase whereas in the whole molecule they are reduced. ULKI simulations demonstrate a tendency to restore the native state, not only where the bridge was attached, but actually all over the molecule.

The rms-positional differences show a similar pattern in the DASI dimer as in the monomer simulations. The time series of the monomer simulations of DASI and native insulin are also in agreement, except for the two segments directly connected to the bridge. Nevertheless shifts are possible, which is, for instance, reflected in the different H-bond pattern.

This overall effect will not change the pattern of deviations among the structure elements. The consequence is that the insulin structure remains intact. Very similar results have been obtained by NMR studies on human proinsulin (Weiss et al. 1990). The reestablished native structure confirms the results found for DASI. The moderate structural perturbation caused by the presence of the bridge can easily be reversed upon its removal.

The accessible surface area

The binding to the receptor can be influenced by the flexibility of the crosslink and by shielding important residues. The following residues have been described as being important for receptor binding (Wood et al. 1975; Pullen et al. 1976; Dodson et al. 1983; Mirmira et al. 1991; Nakagawa and Tager 1992; 1993): in the A chain A1–A5, A19, A21 and in the B chain B12, B16, B22, B24, B25 and B26. In view of the impaired capacity of DASI to bind to the receptor it is of special interest to follow the accessible surface in the course of the simulation. The residues covered by the bridge were identified as follows: For every ps of the DASI monomer simulations the accessible surface area was calculated. The calculations were then repeated after removal of the bridge atoms. Covered residues are revealed upon subtraction of the two calculated areas (see Fig. 9). They are found in close proximity of where the bridge attaches, i.e. of A1, B27, B29. In both molecules residue A19 is shielded. A1, A5 and A15 are also covered in molecule 2. The end of the B chain, which in molecule 1 includes residue B25, is partly covered most of the time,

whereas in molecule 2 B27 is hidden. This means that the bridge not only restricts the motion of the molecule, it can also cover some residues that are relevant for receptor binding.

Conclusion

Irrespective of different resolution of the starting geometries, of the association state and of chemical modification, a number of similarities between DASI, ULKI and native insulin have emerged from the present simulations. The influence of the bridge can be separated from influences of other sources. The main test is the removal of the bridge which results in a movement back towards the native structure. The bridge mainly affects those structure segments to which it is connected. The influence is diverse: it can restrict the mobility of the A_N-helix and the C-terminal B chain, it can shift these segments away from the initial structure and it can modify the fluctuations in the actual insulin moiety.

NMR studies of human insulin exhibit a line broadening of the main-chain amide resonances which was inexplicable on the basis of association phenomena. Comparative investigations of human insulin and des-(B26–B30)-insulin (DPI) revealed the importance of the C-terminal B chain for this line broadening (Hua and Weiss 1991). On the basis of recent NMR studies it has been postulated that insulin exists as a 'molton globule' in its functional form (Hua et al. 1992a, 1993a). Several conformational substances must contribute to this 'molten globule' state.

It is not clear what these conformational substates might look like. One may speculate that the simulated structures with and without the bridge were two possible ones. From NMR studies of proinsulin an influence of the connecting peptide on the insulin moiety can be derived. This is in accordance with the present simulations. In solution even more conformational substates will be populated than seen in the simulations. The C-terminal B chain then adopts the role of a flexible lever with an impact on the whole structure. NMR studies could give indications of whether in DASI the number of possible substates is reduced. Neither D/H exchange nor denaturation experiments show a clear correlation between stability, flexibility and binding (Nakagawa and Tager 1989; Brems et al. 1991). Several factors seem to play a role, one of them being the flexibility of the C-terminal B chain, and another one the interference between the bridge and residues contacting the receptor (Nakagawa and Tager 1986; Mirmira et al. 1991). This theoretical study suggests how the bridge may affect insulin's dynamics and that it prevents the C-terminal B chain from extending away from the body of the molecule. It also indicates that part of the residues involved in receptor interaction may be shielded part of the time. Further studies on other derivatives are encouraged by these results, single-chain insulin being an obvious candidate.

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