3. Materials and Methods

3.1. Bacterial strains and plasmids

Escherichia coli K12 strain SCS1 (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (r_K- m_K+), *supE44*, *relA1*, Stratagene) was used as host for *korB* overexpression plasmids (Balzer *et al.*, 1992). *KorB* overexpression plasmid pMS51-1 and expression vector pMS470D8 have been described (Balzer *et al*., 1992). All the host strains with plasmids overexpressing *korB* or derivatives of *korB* were kindly provided by Dr. E. Lanka (Max Planck Institute for Molecular Genetics, Berlin).

3.2. *E. coli* culture medium (YT medium)

5 gm/l	Yeast extract	
10 gm/l	Tryptone	
5 gm/l	NaCl	

The YT medium was further supplemented with:

25 mM	MOPS pH 8.0(*)	
0.1 % (wt/vol)	Glucose(*)	
25 mg/ml	Thiamine-HCl(*)	
100 µg/ml	Ampicillin (sodium salt)	(sterile filtered)
1 mM	IPTG (for induction)	

(*)autoclaved separately

3.3. FPLC devices and columns (Amersham Biosciences)

ÄKTAexplorer™

Liquid Chromatography Controller LCC-500 PLUS (connected to a recorder and fraction collector)

GradiFrac

HiTrap Heparin Sepharose HP (5 ml)

Mono Q® HR 5/5 (1 ml)

Mono S® HR 5/5 (1 ml)

HiLoad™ 16/60 Superdex™ 75

3.4. Determination of purity

Protein purity is a critical factor in crystallization experiments: proteins used for crystallization should be as pure and homogeneous as possible. The purity of KorB-O protein was visually checked from 18 % SDS polyacrylamide gels (PAGE) stained with Coomassie blue according to standard protocol (Laemmli, 1970).

3.5. Native gel electrophoresis

Under native PAGE conditions, polypeptides retain their higher-order structure and often retain enzymatic activity and the ability to interact with other polypeptides or nucleic acids. The migration of proteins depends on many factors, including size, shape, and native charge. Gels were prepared according to the standard Laemmli SDS protocol but omitting the SDS and the reducing agent (DTT). Samples were not boiled and the gel and tank buffer solutions were without SDS.

3.6. Gel mobility shift assay

Band shift assay also known as electrophoretic mobility shift assay (EMSA) is a useful tool for identifying protein-DNA interactions. The assay can be used to determine the affinity, binding constants and binding specificity of the DNA-binding protein. The assay is performed by incubating the purified protein with oligonucleotides that contain the test binding sequence. The mixture is then separated on a nondenaturing polyacrylamide gel (native gel). Duplexes that are bound by protein migrate more slowly than unbound duplexes and appear as bands that are shifted relative to the bands from the unbound duplexes. The DNA can be biotin-labeled, fluorescent-labeled or radio-labeled for in-gel detection. However the technique can also be utilized using non-labeled oligonucleotides; and was used for investigating the binding of KorB-O to two different DNA duplexes containing the O_B sequence.

3.7. 30-min silver staining method

A simple and rapid protocol (Nesterenko *et al*., 1994) was used for staining protein and double stranded DNA following electrophoresis in a native PAGE. The complete procedure is described in Table 2.

Table 2. Silver staining protocol

Steps[a]	**Solution**	**Time**
Fixation	60 ml acetone stock[b]; 1.5 ml TCA stock[b]; 25 µl 37 % HCHO	5 min
Rinse	ddH_2O	3 × 5 s
Wash	ddH_2O	5 min
Rinse	ddH_2O	3 × 5 s
Pretreat	60 ml actone stock[b]	5 min
Pretreat	100 µl $Na_2S_2O_3.5H_2O$[b] stock in 60 ml ddH_2O	1 min
Rinse	ddH_2O	3 × 5 s
Impregnate	0.8 ml $AgNO_3$ stock[b]; 0.6 ml 37 % HCHO; 60 ml ddH_2O	8 min
Rinse	ddH_2O	2 × 5 s
Develop	1.2 gm Na_2CO_3; 25 µl 37 % HCHO; 25 µl $Na_2S_2O_3.5H_2O$ stock; 60 ml ddH_2O	10-20 s
Stop	1 % glacial acetic acid in ddH_2O	30 s
Rinse	ddH_2O	10 s

[a] All steps performed on a shaker at room temperature. The volumes of all solutions were 60 ml for mini gel 0.75 mm thick.

[b] Stock solutions: 50 % acetone in ddH_2O; 50 % TCA in ddH_2O; 20 % $AgNO_3$ in ddH_2O (store in dark); 10 % $Na_2S_2O_3.5H_2O$ in ddH_2O

3.8. Expression and purification of DNA binding domain of KorB (KorB-O)

Bacterial cultures of SCS1 (pMS51-12) were grown in a shaker at 37ºC in 1 litre of YT medium. Gene expression was induced at OD_{600} ~ 0.5 by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to the culture and shaking continued for ~ 4 hours. Cells were harvested (4,000×g, 10 min) and resuspended in 20 mls of resuspension buffer (200 mM NaCl, 2 mM EDTA and 30 mM Tris-HCl pH 7.6). Cell lysis was done using a

French Press and the resulting extract was centrifuged (19,000 rpm, 60 min) to remove the cellular debris.

The operator-binding domain of KorB (KorB-O) was purified in a four-step procedure as described below and shown in Fig. 5.

STEP I: ***DEAE-Sephacel chromatography***

The supernatant was diluted in 50 ml ddH_2O, passed through a 0.2 μ filter and loaded onto a DEAE-Sephacel column equilibrated with 100 ml of Buffer A (1 mM EDTA, 1 mM DTT, 50 mM NaCl and 20 mM Tris-HCl pH 7.6). The column was washed with 200 ml of Buffer A and KorB-O was eluted (Peak 1, Fig. 5) by running a linear gradient of 50 to 600 mM NaCl over 300 ml at a flow rate of 1.5 ml/min. KorB-O protein eluted at around 200 mM NaCl.

STEP II: ***Heparin-Sepharose chromatography***

From the pooled fractions 15 ml were diluted with 35 ml of Buffer B (1 mM EDTA, 1 mM DTT, and 20 mM Tris-HCl pH 7.6) and loaded to a HiTrap Heparin column (5 ml). A gradient of 50 to 600 mM NaCl over 150 ml was run at a flow rate of 5 ml/min and 2 ml fractions were collected.

STEP III: ***Anion-exchange chromatography***

MonoQ HR 5/5 was used in the third step at 1 ml/min with the same gradient but over 30 ml and collecting 0.5 ml fractions.

STEP IV: ***Gel-filtration chromatography***

Final polishing was done on a HiLoad 16/60 Superdex 75 (Pharmacia) equilibrated with 50 mM NaCl and 20 mM Tris pH 7.6 and at a flow rate of 1 ml/min. KorB-O migrates as a single peak eluting at ~ 30 kDa (the calculated molecular mass of the monomer being 19.9 kDa).

The KorB-O protein was further concentrated to 2.4 mg/ml in a 5K cutoff Ultrafree Millipore membrane. Protein concentration was determined by measuring OD_{280} and taking into account a molecular weight of 19,919.9 Da and a molar extinction coefficient of 13940 M^{-1} cm^{-1} (determined by ProtParm Tool, Expasy server http://us.expasy.org/).

The protein encoded by pMS51-12 (E101-K294) degrades to a stable fragment named KorB-O consisting of residues R117-K294 as confirmed by N-terminal sequencing and mass spectrometry. KorB-O has a theoretical pI of 4.73.

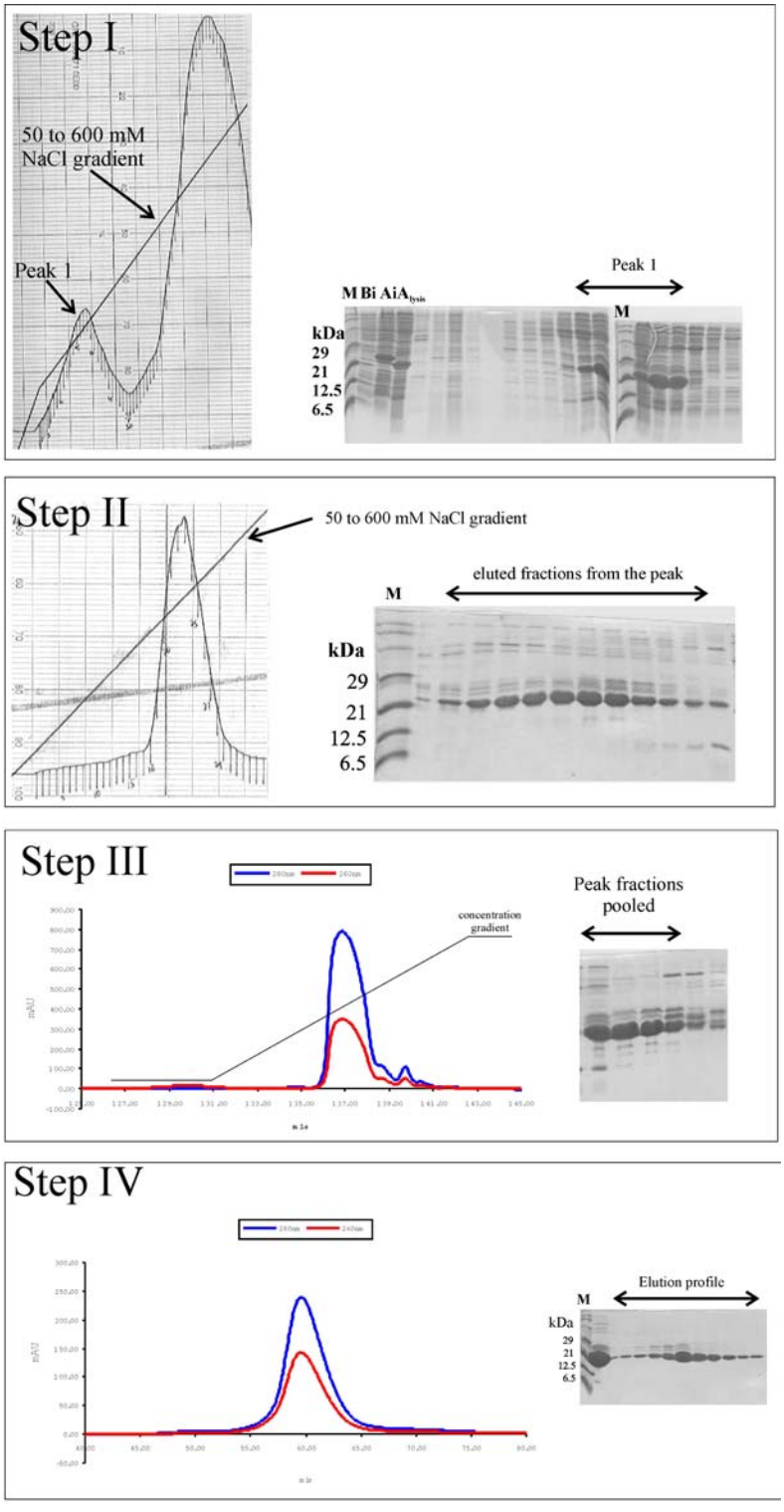

Figure 5. **Purification of KorB-O**. Chromatograms show the elution profile from the four steps of purification and the SDS PAGE gels are from the eluted fractions (M: marker, Bi: before induction, Ai: after induction and A_{lysis}: after cell lysis). The procedure included DEAE-Sephacel (Step I), Heparin Sepharose (Step II), Anion exchange (Step III) and Gel-filtration (Step IV) chromatography steps.

3.9. Preparation of oligonucleotides for crystallization

Single stranded HPLC-purified (bromo-substituted) oligonucleotides for crystallization were chemically synthesized by Biotez, Berlin. Equimolar amounts of complementary strands each at a concentration of 250 pmol/µl were prepared in 50 mM NaCl and 20 mM Tris pH 7.6. These mixtures were then heated to 96 °C in a large water bath, which was then allowed to cool to room temperature over night. The resulting duplexes were further purified over a gel-filtration column Superdex 75, equilibrated in 20 mM Tris-HCl buffer, pH 7.6 and 50 mM NaCl (Fig. 6). Small amounts were run on a native acrylamide gel to check for the purity of the annealed duplex.

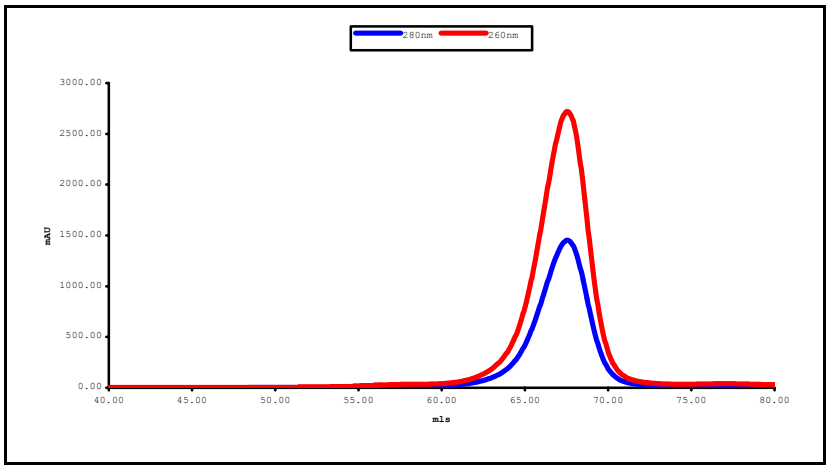

Figure 6. Elution profile of O_B. The DNA duplex was passed through the gel-filtration column Superdex 75 at a flow rate of 1 ml/min. The peak fractions were pooled and concentrated in a 5K cutoff Ultrafree Millipore membrane.

The concentration of the duplex was calculated based a molecular weight of 10,540 Da and a molar extinction coefficient of 271222 $M^{-1}cm^{-1}$, which was corrected for the hyperchromic effect.

3.10. Chemical crosslinking

The KorB-O protein (2.4 mg/ml) and the KorB-O—O_B complex (0.5 mg/ml) were treated with increasing concentrations of glutardialdehyde and incubated for 1 hour at room temperature. 1µl of KorB-O and 5 µl of KorB-O—O_B were mixed with 9 µl and 5 µl of glutaraldehyde of various concentrations, respectively. The reaction was stopped by adding

4 µl of 1 M Glycin. 4 µl of loading buffer was added to the samples (no boiling) and SDS PAGE was carried out and the gel subsequently stained with Coomassie solution.

3.11. Crystallization experiments

The starting point for any X-ray crystallographic investigation on protein-nucleic interaction is the growth of suitable well diffracting protein-DNA crystals. Initial crystallization conditions were found using Crystal Screen™ and Crystal Screen 2™ (both from Hampton Reasearch, Aliso Viejo, USA). Crystal Screen™ utilizes the original sparse matrix screen (Jancarik & Kim, 1991) and evaluates 50 combinations of pH, salt, precipitants for their ability to promote growth of crystals. Crystal Screen 2™ is an extension (Cudney *et al*., 1994) of the sparse matrix screen and utilizes 48 further combinations of reagents.

Two of the most commonly used methods for protein crystallization fall under the category of vapour diffusion. These are known as the **hanging drop** and **sitting drop** methods.

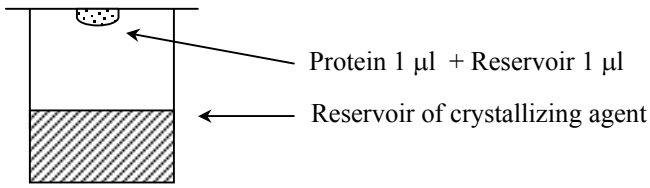

Figure 7. Schematic representation of hanging drop experiment. Reservoir solution (hatched lines) contains buffer and precipitant. Protein solution (dotted drop) contains the same compounds, but in lower concentrations.

Both entail a droplet containing purified protein, buffer, and precipitant being allowed to equilibrate with a larger reservoir containing similar buffers and precipitants in higher concentrations. The hanging drop vapour diffusion method as shown in Fig. 7 is one of the most popular methods for crystallization of macromolecules and was used throughout the present work. The principle is quite simple. A small volume of the protein sample is placed in the centre of a siliconized cover slide and mixed with an equal volume of the precipitant. The cover slip is inverted quickly and placed onto a well of a 24-well Linbro plate filled with the precipitant and the rim pregreased. Water vapours leave the drop and the sample

undergoes a relative increase in supersaturation. Equilibration is reached when the reagent concentration in the drop is approximately the same as that in the reservoir.

3.12. Data collection and processing

For cryogenic data collection all crystals were mounted in Copper cryo loops and flash frozen in liquid nitrogen prior to the X-ray diffraction experiment. Crystals were first tested for usable diffraction at the home source at 110 K using a Rigaku RU H2B rotating anode X-ray generator producing CuKα radiation. The generator was run at 44 kV, 110 mA with a 0.3 mm focus. A crystal which diffracted to ~ 3 Å was frozen back into liquid nitrogen and complete MAD data to 2.2 Å resolution was collected at the ID 14.2 beamline at BESSY, Berlin. All data sets were reduced with the program XDS (**X**-Ray **D**etector **S**oftware) (Kabsch, 1993).

3.13. Programs and software used

Data processing and reduction

XDS (Kabsch, 1993), CHOOCH (Evans and Pettifer, 2001)

Data modification programs

CCP4 (CCP4, 1994)

Automated localization of heavy atoms

SOLVE (Terwilliger and Berendzen, 1999)

RESOLVE (Terwilliger, 2000)

Model building

O (Jones *et al*., 1991), ARP/wARP (Perrakis *et al*., 2001)

Refinement

REFMAC5 (CCP4, 1994)

Figures

Molmol (Koradi *et al*., 1996)

Image rendering

POV-ray (www.povray.org)

Electrostatic Surface potentials

GRASP (Nicholls *et al*., 1993)

Linkage plot

XSAE

Structure check

Procheck (Laskowski *et al*., 1993)

Whatcheck (Hooft *et al*., 1996)

PROMOTIF (Hutchinson and Thornton, 1994)

SFCHECK (Vaguine *et al*., 1999)