# Characterization of the α and β Subunits of Casein Kinase 2 by Far-UV CD Spectroscopy†

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ABSTRACT: Although Chou–Fasman calculations of the secondary structure of recombinant casein kinase 2 subunits α and β suggest they have a similar overall conformation, circular dichroism (CD) studies show that substantial differences in the conformation of the two subunits exist. In addition, comparison of the far-UV CD spectrum of reconstituted CK-2 with the spectra of the subunits indicates that conformational changes occur in the backbone region upon association. Such changes may explain the increased enzyme activity of the holoenzyme relative to that of the α subunit itself. In contrast, no changes in the far-UV CD spectrum of the α subunit are observed in the presence of casein or the synthetic decapeptide substrate RRRDDDSDDD. Furthermore, the α-helical structure of the α subunit (but not the β subunit) can be increased in the presence of stoichiometric amounts of heparin, presumably by its binding to the polylysine stretch at amino acid positions 74–77. Heat denaturation experiments (25–90 °C) support the notion that heparin may provide a local protective function. A similar but much larger effect was also observed in the presence of the β subunit only, which supports previous suggestions of a protective function for this subunit. These results indicate that the protection provided by the β subunit and the increased enzyme activity of the holoenzyme may arise, in part, from a stabilization of the conformation of the enzyme complex and an increase in α-helical content.

Casein kinase 2 (CK-2)[1] is a pleiotropic growth-related protein kinase (Rose et al., 1981; Prowald et al., 1984; Schneider et al., 1986; Seitz et al., 1989; Rydell et al., 1990; Lamprecht et al., 1990; Münstermann et al., 1990; Pistorius et al., 1991). However, the mechanisms for the observed constitutive expression in rapidly proliferating tissue, compared to "normal" growing cells, have yet to be elucidated. Whether this is due to changes at the transcriptional level involving the CK-2 regulatory domain or whether transcriptional factors are involved is an open question and must await the elucidation of the genomic structure of CK-2. First steps in this direction are being made now, since the enzyme has been cloned and suitable probes are available. Parallel to these efforts, the availability of cDNAs allowed the expression and characterization of recombinant CK-2 α and β subunits. Successful expression of CK-2 subunits was described for the α subunit (Hu & Rubin, 1990), for a fusion protein of the β subunit (Boldyreff et al., 1991), and for the α and β subunits (Grankowski et al., 1991).

Native CK-2 exhibits a tetrameric structure of the $\alpha_2\beta_2$ type [see Pinna (1990) and references cited therein] or an $\alpha\alpha'\beta_2$ type (Litchfield et al., 1990; Lozeman et al., 1990; Maridor et al., 1991). In contrast to A-kinase ($R_2C_2$) which also has a tetrameric structure, the CK-2 holoenzyme is not dissociated by a specific agonist, in a manner analogous to the cAMP-induced dissociations seen with the A-kinase. Previous studies have suggested that the α subunit carries the catalytic domain for the kinase function of CK-2 (Hathaway et al., 1981; Feige et al., 1983; Grankowski et al., 1991). The function of the β subunit, however, remained obscure, since it cannot be isolated from the holoenzyme under nondenaturing conditions. Hence, the CK-2 molecule is unique in its makeup among the known Ser/Thr kinases (Taylor, 1991).

Sequence analysis of the β subunit revealed that it is somewhat related to a proteinaceous star-shaped structure in primary spermatocytes from *Drosophila*, a finding which does not necessarily shed more light on its biological role in the CK-2 holoenzyme (Livak, 1990). Even the availability of the holoenzyme in large quantities was of little help in the elucidation of the functional role of the individual subunits of the CK-2 molecule. Obviously, only substantial amounts of purified α and β subunits would permit the necessary biochemical and biophysical experiments to address this question.

We have expressed the cDNAs from the α and β subunits in *Escherichia coli* (Grankowski et al., 1991). From the biochemical studies, the α subunit does indeed exhibit the properties of a Ser/Thr kinase, confirming earlier observations. The β subunit by itself shows no kinase activity. However, the β subunit can stimulate the kinase activity of the α subunit maximally when added in stoichiometric amounts (Grankowski et al., 1991). Furthermore, it was shown that the β subunit also exhibits a protective function (Meggio et al., 1992), since it helps to maintain kinase activity even when denaturing conditions prevail (e.g., in the presence of urea, high temperature, proteolysis, etc.). This latter observation concerning the stabilizing properties of the β subunit prompted our interest in examining the secondary structure of the individual CK-2 α and β subunits and the effect of their reconstitution on the resulting structure of the holoenzyme. In addition, the binding

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[1] Abbreviations: CK-2, casein kinase 2; rCK-2, recombinant casein kinase 2; CD, circular dichroism; PMSF, phenylmethanesulfonyl fluoride.

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of substrates or inhibitors to the individual subunits and/or holoenzyme may result in conformational changes which will provide additional clues to their functional role(s).

## Materials and Methods

*UV Spectra.* UV spectra were acquired on an AVIV Model 118DS spectrometer in 4-mm cuvettes at room temperature. Protein concentration was about 1 mg/mL in 20 mM phosphate buffer, pH 7.4. Spectra were recorded with a 0.5-nm step and a time constant of 1 s.

*Circular Dichroism.* CD studies were performed with an AVIV Model 62DS spectropolarimeter and circular cells with a 0.2-mm path length. Samples were prepared in 20 mM phosphate buffer, pH 7.4, at a concentration of about 20 $\mu$M. Spectra were recorded using a 1.5-nm bandwith, a 0.5-nm step, and a time constant of 4 s. A total of four scans were averaged for both sample and solvent. After correction of the sample spectrum for solvent and cell contributions, the data were fit by nonlinear regression analysis. The instrument was calibrated by using (+)-10-camphorsulfonic acid (Tuzimura et al., 1977).

Melting curves were obtained using a thermoelectric cell holder and monitoring the CD intensities at 260, 222, and 206 nm in 1 °C increments from 25 to 90 °C. The temperature equilibration time was 1 min with a time constant of 10 s. The data were corrected for base-line shifts and possible concentration changes.

*Secondary Structure Estimation.* Estimation of the secondary structure for the CD data, expressed as mean residual ellipticity, was made with the program CONTIN (v.2, Provencher & Glöckner, 1981). This method uses reference spectra deduced from 16 proteins, the structures of which are known from X-ray crystallography (Provencher & Glöckner, 1981).

*Secondary Structure Prediction.* Predictions of the secondary structure were made according to the algorithms of Chou and Fasman (1977) using the programs MSEQ (MSEQ, v1.17, University of Michigan Software, Ann Arbor, MI) and of Garnier et al. (1978) with the program PEPTIDE STRUCTURE. After automatic assignment of the secondary structure by the programs, minor adjustments were made by visual inspection of the sequence (e.g., a single amino acid labeled as "$\alpha$-helical" might be assigned to the next most probable structure).

*Stokes Radius Analysis.* Stokes radii were determined using a Sephacryl S-200 (Pharmacia) column (0.8 × 55 cm) equilibrated with PI-1500 buffer (20 mM Tris-HCl, pH 8.15, containing 0.5 mM DTE, 1.5 M NaCl, and 0.5 mM PMSF). Samples containing 2 mg of aldolase, 2 mg of transferrin-holo, 2 mg of cytochrome *c* (all from Boehringer-Mannheim), and 100 $\mu$g of either rCK-2, rCK-2 $\alpha$, or rCK-2 $\beta$ in 0.5 mL were applied and the proteins fractionated at a flow rate of 10 mL/h. Elution of the proteins was monitored by (1) the absorbance at 280 nm, (2) immunoblotting using the anti-CK-2 antibodies, and (3) CK-2 activity measurements. Points are the mean value of at least three independent determinations.

*Sucrose Density Gradient Sedimentation.* A linear gradient (13 mL) of sucrose (5–25% w/w) was prepared in PI-300 buffer (20 mM sodium phosphate buffer, pH 8.15, containing 0.5 M DTE, 300 mM NaCl, and 0.5 M PMSF) and also in the absence of salt. Protein samples containing 2 mg of aldolase, 2 mg of ovalbumin, 2 mg of cytochrome *c*, and 100 $\mu$g of either rCK-2, rCK-2 $\alpha$, or rCK-2 $\beta$ were loaded on the top of the gradient and centrifuged at 200000*g* in a Kontron TST 41 rotor for 26 h at 4 °C. After centrifugation, fractions (0.75 mL) were collected and monitored by absorbance, immunoblotting, and enzyme activity, as noted above. All points are the mean value of at least three independent determinations.

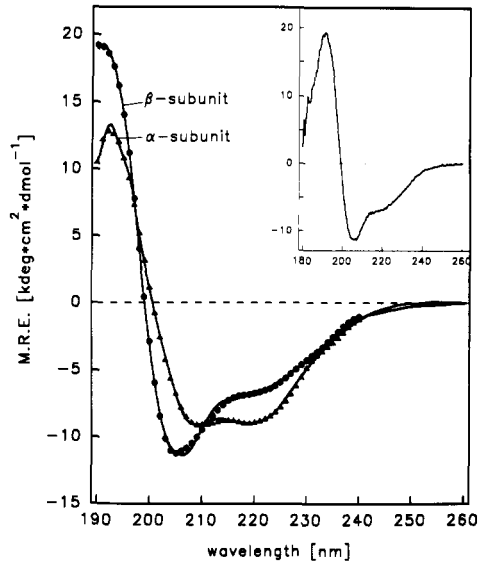


FIGURE 1: Far-UV CD spectra of recombinant CK-2 subunits $\alpha$ (▲) and $\beta$ (●) in 20 mM phosphate buffer, pH 7.4 at 25 °C, and the computer fit of the CD spectra. Insert: raw data for the $\beta$ subunit prior to curve fitting.

*Cloning and Expression of CK-2 Subunits.* The cloning and expression of the subunits were carried out as described earlier (Grankowski et al., 1991) using the pT7-7-derived vectors pBB-3 ($\alpha$ subunit) and pBB-4 ($\beta$ subunit). Purification of the subunits was somewhat modified compared to the original procedure: 10–15 g of *E. coli* cells was suspended in distilled water containing 1 mM PMSF. The cells were incubated for 1 h in an ice bath, followed by sonication treatment (usually two to three cycles with 10-min intervals on ice). After sonication, the bacterial extract was centrifuged for 10 min at 30000*g*. The $\alpha$ subunit was obtained from the supernatant and the $\beta$ subunit from the pellet. Mixing of stoichiometric amounts of $\alpha$ and $\beta$ subunits led to reconstitution of the complex. Alternatively, recombinant CK-2 holoenzyme was prepared by mixing bacteria which express either the $\alpha$ or the $\beta$ subunit, the combined bacteria were then lysed, and the recombinant holoenzyme was isolated as described for the $\beta$ subunit (Grankowski et al., 1991). Recombinant holoenzyme was shown earlier to be indistinguishable by biochemical and biophysical means from the native holoenzyme (Grankowski et al., 1991). In addition, the CD spectrum of the native holoenzyme from rat liver showed no significant differences from the CD spectrum of the recombinant reconstituted enzyme (data not shown).

*Protein Determination.* The amount of protein in the samples was determined according to Bradford (1976) using the commercially available Bio-Rad reagent. In addition, the molar extinction coefficients of $\epsilon_{279}$ = 60 495 $M^{-1}$ $cm^{-1}$ and $\epsilon_{279}$ = 29 165 $M^{-1}$ $cm^{-1}$ for the $\alpha$ and $\beta$ subunit, respectively, were in good agreement (±5%) with that calculated from their amino acid composition (Gill & von Hippel, 1989).

## Results

*CD Spectra of CK-2 Subunits.* The far-UV CD spectrum of the $\alpha$ and $\beta$ subunits of CK-2 in 20 mM phosphate buffer, pH 7.4 and 25 °C, is shown in Figure 1. The quality of the spectra was very good, as can be seen for the $\beta$ subunit in the insert. Although analysis of the CD spectra by the CONTIN program used the data after a nonlinear fit, the signal to noise

Table I: Fraction of Secondary Structure of CK-2 and Subunits

| | α-helix | β-sheet | remainder[a] |
|---|---|---|---|
| α subunit | | | |
| CD[b] | 0.30 | 0.38 | 0.32 |
| Chou–Fasman | 0.24 | 0.26 | 0.50 |
| β subunit | | | |
| CD | 0.26 | 0.59 | 0.15 |
| Chou–Fasman | 0.23 | 0.26 | 0.51 |
| CK-2 enzyme | | | |
| CD | 0.32 | 0.45 | 0.23 |

[a] β-Turn and random coil. [b] CONTIN program estimates.

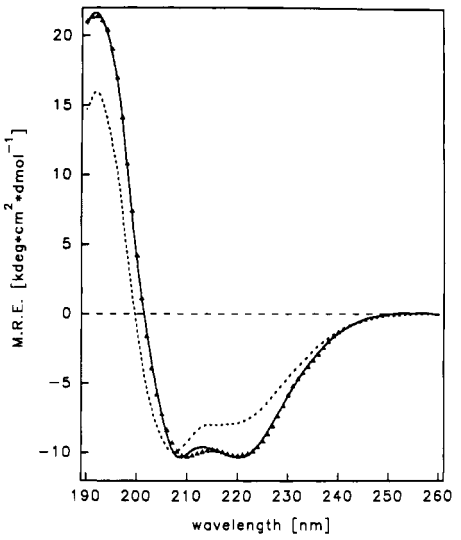


FIGURE 2: CD spectrum of the reconstituted CK-2 enzyme (experimental; —), fit of the CONTIN analysis (▲—▲), and the calculated spectrum (---) obtained by summation of the two-subunit CD spectra shown in Figure 1.

was high, even deep into the far-UV. The computer-derived fit of the data using the CONTIN program for the α and β subunits shown in Figure 1 is in good agreement with the experimental spectra. The α subunit has a higher proportion of α-helical structure than the β subunit, as evidenced by the two strong, negative bands at 209 and 222 nm of nearly equal intensity. In contrast, the β subunit is characterized by a very high proportion of β-sheet structure (59%) and displays a comparatively weak band at 222 nm, an intense negative band at 206 nm, and a strong positive band at 191 nm. The fraction of α-helix, β-sheet, and remainder (β-turn and undefined) for each subunit as estimated from the CD data by the CONTIN program is given in Table I.

*CD Spectrum of Reconstituted CK-2.* The far-UV CD spectrum of the reconstituted CK-2 enzyme in 20 mM phosphate buffer, pH 7.4 at 25 °C, is shown in Figure 2 along with the computer fit of the data. The CD spectrum of the recombinant reconstituted CK-2 enzyme is identical to that of the native holoenzyme from rat liver (data not shown). The spectrum is characterized by prominent minima at 209 and 222 nm and a strong positive band at 191 nm. The computer assignment of the secondary structure is given in Table I. Also shown in Figure 2 is the calculated spectrum of the reconstituted CK-2 enzyme, obtained as the average of the individual component CD spectra shown in Figure 1. Substantial differences exist between the experimental spectrum and that calculated from the individual subunits. The mean residue ellipticity of both the positive and negative bands is greater in the experimental spectrum, and the negative band at 222 nm is similar in intensity to that of the shorter wavelength band at about 207 nm.

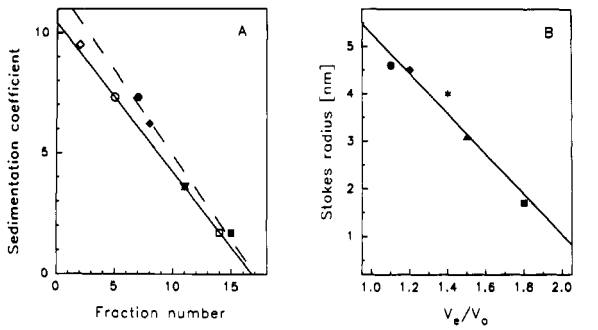


FIGURE 3: (A) Estimation of the sedimentation coefficients for rCK-2 and its subunits. Protein standards [aldolase, 7.3 S (●); cytochrome *c*, 1.7 S (■); ovalbumin, 3.6 S (▼)], rCK-2 (♦), and subunits (▲) were centrifuged in a 5–25% sucrose gradient. Open symbols are values obtained in the absence of salt. (B) Estimation of the Stokes radius for rCK-2 and its subunits. Gel filtration was performed on a Sephacryl S-200 column calibrated with aldolase [*s* = 4.6 nm (●)], transferrin [*s* = 4 nm (*)], and cytochrome *c* [*s* = 1.7 nm (■)]. The position of CK-2 (♦) and its subunits (▲) is as shown. $V_e$ is the elution volume of the protein, and $V_0$ is the void volume of the column (15 mL).

*Sucrose Density Gradient Sedimentation.* The sedimentation coefficient of the holoenzyme in the presence and absence of 0.3 M NaCl is shown in Figure 3A. The value of 6.2 S is in good agreement with that published previously by Hu and Rubin (1990) (6.5 S) and Glover et al. (1986) (6.0 S). In the absence of salt (20 mM sodium phosphate buffer, pH 8.15), the sedimentation value for the holoenzyme increased drastically to 9.5 S, and about 30% of the total material precipitated. This is only a rough estimate obtained by comparison of the material seen on the immunoblots. In contrast, the *s* values for the α and β subunits (3.4 S) did not show such a salt dependency, and only very slight shifts to lower *s* values were observed even at high salt concentrations (Figure 3A).

*Stokes Radii.* The Stokes radii for the α subunit, β subunit, and the holoenzyme in high-salt PI-1500 buffer are shown in Figure 3B. For the α subunit and the holoenzyme, the values of 3.1 and 4.5 nm, respectively, are close to those obtained in the presence of 2 M KCl by Hu and Rubin (1990) for nematode *Caenorhabitis elegans* CK-2 α subunit (2.8 nm) and holoenzyme (5.0 nm). The recombinant CK-2 β subunit had virtually the same Stokes radius as the α subunit, in agreement with the results obtained by sedimentation analysis. At lower salt concentrations, no distinct elution profile could be obtained for either the α subunit or the holoenzyme. The elution pattern of the β subunit was not affected, however. Similar Stokes radii were obtained for the β subunit in 6 M urea and 14 mM β-mercaptoethanol, further ruling out dimer formation.

*CD Spectra in the Presence of Heparin.* The addition of an equimolar amount of heparin to the α subunit in 20 mM phosphate buffer, pH 7.4 at 25 °C, leads to distinctive changes in the CD spectrum of the protein, as shown in Figure 4a. Although heparin itself does not give a CD signal, the intensity of both the negative band at 222 nm and the positive band at about 190 nm for the α subunit increases and approaches that observed for the reconstituted CK-2 enzyme. This is in contrast to both the reconstituted CK-2 enzyme (data not shown) and the β subunit (Figure 4b) where no changes in CD intensity or band shape are observed when a stoichiometric amount of heparin is added.

*CD Spectra in the Presence of Casein and a Synthetic Substrate Peptide.* No change in the far-UV CD spectra of the α and β subunits in the presence of 0.6–2.4 μM casein was observed. Similarly, the synthetic decapeptide substrate

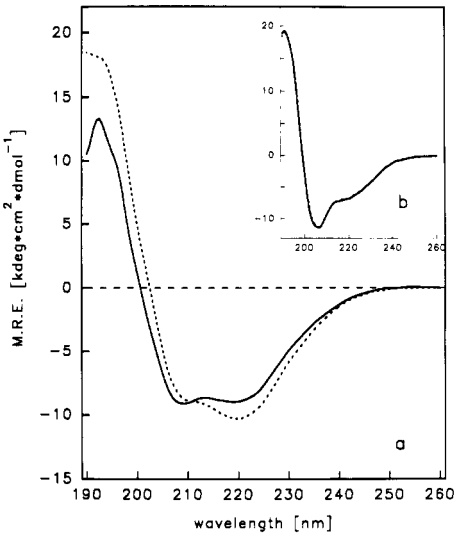


FIGURE 4: CD spectra of the $\alpha$ (a) and $\beta$ (b) subunits in the presence (---) and absence (—) of equimolar amounts of heparin. Heparin alters the CD spectrum of the $\alpha$ subunit only.

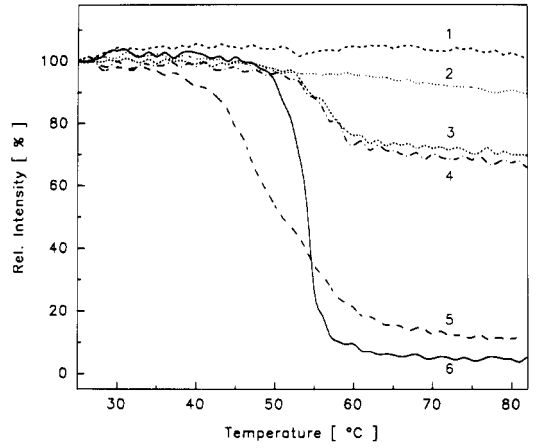


FIGURE 5: Melting curves for CK-2 holoenzyme (222 nm) in the presence (1) and absence (6) of heparin, for $\alpha$ subunit (222 nm) in the presence (2) and absence (5) of heparin, and for $\beta$ subunit (206 nm) in the presence (3) and absence (4) of heparin.

RRRDDDSDDD at concentrations of 30–300 $\mu$M had no effect (results not shown).

*Temperature Effects.* The melting curve for the $\alpha$ subunit of CK-2 between 25 and 80 °C is shown in Figure 5, curve 5. The CD spectrum remains relatively constant until about 40 °C, after which there are rapid and irreversible changes indicative of protein denaturation. Between 55 and 60 °C, there is little CD signal remaining above 210 nm. In the presence of a stoichiometric amount of heparin (Figure 5, curve 2), the $\alpha$ subunit still undergoes subtle irreversible changes, although at a greatly reduced rate. Even at 80 °C, a substantial signal can still be observed at 222 nm. Similar melting curves are shown for the reconstituted CK-2 enzyme and the $\beta$ subunit. Compared to the $\alpha$ subunit, the reconstituted enzyme is much more resistant to heat-induced denaturation, with a loss of structure occurring rapidly only above 55 °C (Figure 5, curve 6). In the presence of heparin, CK-2 enzyme shows a small but measurable increase in resistance to heat denaturation. Thus, heparin and the $\beta$ subunit both give a degree of thermal stability to the $\alpha$ subunit. In contrast, heparin has no effect on the melting curve for the $\beta$ subunit. The $\beta$ subunit begins to denature at about 53 °C and reaches a stable structure around 60 °C. The heat-induced structural changes in the $\beta$ subunit were not reversible.

## Discussion

Although Chou–Fasman analysis of the secondary structure of recombinant CK-2 subunits $\alpha$ and $\beta$ suggests they have a similar overall conformation (Table I), the CD spectra show that their conformations differ substantially. In particular, the proportion of $\alpha$-helical structure in the $\alpha$ subunit is higher than that found in the $\beta$ subunit, which is instead dominated by a high percentage of $\beta$-sheet conformation.

The hydrodynamic properties of the recombinant proteins confirm a monomeric and folded structure for both the $\alpha$ and $\beta$ subunits. In the presence of salt, a sedimentation value of 3.4 S was obtained for both subunits, which was only slightly shifted to higher values in the absence of NaCl. Thus, neither the CK-2 $\alpha$ nor the CK-2 $\beta$ subunit showed a tendency to self-aggregate in salt-free buffers. In the presence of salt, the Stokes radii were also nearly identical for both subunits, which eluted at the same fraction from the Sephacryl column. These results are surprising since the $\beta$ subunit has only half the molecular mass of the $\alpha$ subunit and suggests that the shape of the molecule deviates from the assumed globular structure. However, there are examples of low molecular mass proteins (e.g., histone H1, MW 23 000) which exhibit relatively high Stokes radii (4.2 nm). At low salt concentrations, the elution profile of the $\beta$ subunit was unchanged while no distinct profile could be obtained for the $\alpha$ subunit. This is in agreement with previous observations (Hu & Rubin, 1990) that the $\alpha$ subunit binds to Sephacryl beads under low-salt conditions.

Reconstitution of recombinant CK-2 results in an enzyme whose CD spectrum is different from the sum of its components. The minimum and maximum mean residue ellipticities of the experimental spectrum are greater than those of the calculated spectrum, indicating that reconstitution results in an increase in the structure of the enzyme. In addition, the similar intensity of the minima at 209 and 222 nm in the CD spectrum of the reconstituted enzyme contrasts sharply with the weak shoulder of the calculated spectrum at 222 nm. The $\alpha$-helical content calculated from the fit of the CD spectrum is highest in the reconstituted enzyme, suggesting that reconstitution results in an increase and/or stabilization of the $\alpha$-helical character of one or both of the subunits. This may correlate with the increased enzyme activity of the holoenzyme relative to that of the $\alpha$ subunit itself.

The hydrodynamic properties of the reconstituted enzyme are also somewhat different from those of its subunits. In a detailed study, Glover (1986) has shown that the sedimentation coefficient of *Drosophila* CK-2 was dependent on the ionic strength and the enzyme concentration. As the salt concentration was reduced below 200 mM, the enzyme self-aggregated. Our results are consistent with these observations. We observed that the sedimentation coefficient increases from 6.2 to 9.5 S as the salt concentration is lowered and much of the material precipitates.

For the $\alpha$ subunit, a change is observed in the CD spectrum when an equimolar amount of the inhibitor heparin is added. An overall increase in structure of the $\alpha$ subunit occurs, which is reflected as an increase in signal intensity, especially below 200 nm. The minimum at 222 nm is also more intense in the presence of heparin, suggesting an increase in and/or stabilization of the $\alpha$-subunit helical content. A similar increase in structure of the reconstituted holoenzyme in the presence of heparin is not observed, although the enzyme displays a small increase in resistance to thermal denaturation. As reconstitution of the enzyme results in a significant increase in $\alpha$-helical content, it is possible that the conformational changes

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1   MSGPVPSRAR VYTDVNTHRP REYWDYESHV VEWGNQDDYQ LVRKLGRGKY
    ttrtrttrrß ------t--- ------ßttß ßt-----ß-- ----t---α-

51  SEVFEAINIT NNEKVVVKIL KPVKKKKIKR EIKILENLRG GPNIITLADI
    -------ßßß α--------- rrα------- -----rrt-- -ß-------r

101 VKDPVSRTPA LVFEHVNNTD FKQLYQTLTD YDIRFYMYEI LKALDYCHSM
    tttßttatßt ttßßt---ßt ß-------t- --ßtßα---- ---ßtr----

151 GIMHRDVKPH NVMIDHEHRK LRLIDWGLAE FYHPGQEYNV RVASRYFKGP
    ttrrarrttt ßßα------- -ßßrrα---- -rt---ß--- ------t---

201 ELLVDYQMYD YSLDMWSLGC MLASMIFRKE PFFHGHDNYD QLVRIAKVLG
    ß---------- ---t---ßßα ---------- rrrt---ß-- --------rr

251 TEDLYDYIDK YNIELDPRFN DILGRHSRKR WERFVHSENQ HLVSPEALDF
    ααrß------ --arrt---ß rßrt---ßtt ααßßtt---r tttrα-----

301 LDKLLRYDHQ SRLTAREAME HPYFYTVVKD QARMGSSSMP GCSTPVSSAN
    ----ßrtttr tα------rr ß------α-- --t------- -------α--

351 MMSGISSVPT PSPLGPLAGS PVIAAANPLG MPVPAAAGAQ Q
    -tttrtrt-- -----rtttß ßααarrt--- rrα-----rr t
```

FIGURE 6: Secondary structure prediction for the $\alpha$ subunit of CK-2. $\alpha$, $\beta$, t, and r represent $\alpha$-helix, $\beta$-sheet, turn, and random coil, respectively. The dashed line indicates a string of four or more amino acids with the same predicted secondary structure. Underlined are the conserved Gly-X-Gly-XX-Ser component of the NTP binding site at residues 45–51, the putative heparin binding site Val-Lys-Lys-Lys-Lys at residues 73–77, and $Lys^{68}$, which is known to be essential for expression of catalytic activity.

associated with heparin binding to the $\alpha$ subunit alone are no longer possible in the holoenzyme. Alternatively, a small increase in $\alpha$-helical content may occur, but it is masked by the already large minimum at 222 nm present in the enzyme complex.

It has been shown that replacement of lysines-74/75 in the $\alpha$ subunit by glutamic acid residues leads to an almost complete loss of CK-2 inhibition by heparin (Hu & Rubin, 1990). This region of the $\alpha$ subunit, containing the tetralysine stretch 73–76, has been suggested as the heparin binding site that leads to a highly specific inhibition of CK-2 activity (Hathaway et al., 1980; Hu & Rubin, 1990). While Chou–Fasman and GOR secondary structure predictions both underestimate the percentage of helical structure in the $\alpha$ subunit, what is assigned as $\alpha$-helical includes this region of the subunit (Figure 6). A recent X-ray crystal structure of the catalytic subunit of cAMP-dependent protein kinase (Knighton et al., 1991) also finds an $\alpha$-helical stretch in the analogous region of this enzyme. Our results indicate that the binding of heparin to this tetralysine region, which is only a few residues removed from the critical $Lys^{68}$ catalytic site and is also near the conserved GXGXXS NTP binding site (Figure 6), changes the backbone conformation of the $\alpha$ subunit and accounts for inhibition of the enzyme by heparin binding.

The X-ray crystal structure of the catalytic subunit of cAMP-dependent protein kinase has the substrate binding domain located in the large, predominantly helical lobe of this subunit. The catalytic domains within the protein kinase family are highly conserved (Hanks et al., 1988), and the $\alpha$-helical content of the cAMP-dependent protein kinase catalytic subunit (32%) is very similar to that obtained in this study for the $\alpha$ subunit of CK-2 (Table I). Although we cannot assign the conformational changes that occur upon reconstitution solely to the $\alpha$ subunit, we speculate that the increase in $\alpha$-helical character is predominantly associated with a conformational change in the $\alpha$ subunit. That no additional increase in the helical character of the holoenzyme is observed in the presence of heparin may indicate a maximal effect by the $\beta$ subunit.

The heat denaturation experiments also lend support for a direct interaction of heparin with the $\alpha$ subunit as a distinct stabilizing effect was observed in the presence of the inhibitor. A similar but much larger effect was observed in the presence of the $\beta$ subunit only, which supports previous suggestions of a protective function for this subunit (Meggio et al., 1992). The results from this study indicate that this protection results from a stabilization of the conformation of the enzyme complex and an increase in $\alpha$-helical content.

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# Functional Analysis of Chimeric Proteins Constructed by Exchanging Homologous Domains of Two P-Glycoproteins Conferring Distinct Drug Resistance Profiles†

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ABSTRACT: P-Glycoproteins (P-gps) encoded by the mouse *mdr1* and *mdr3* (Phe$^{939}$, *mdr3F*) genes confer distinct drug resistance profiles. While the *mdr1* and and *mdr3F* clones confer comparable levels of vinblastine (VBL) resistance, *mdr3F* confers actinomycin D (ACT) resistance levels 2-fold greater than *mdr1*, while *mdr1* confers resistance to colchicine at levels 7-fold greater than *mdr3F*. We wished to identify in chimeric proteins discrete protein domains responsible for the distinct drug resistance profiles of *mdr1* and *mdr3F*. Homologous protein domains were exchanged in hybrid cDNA clones, and the specific drug resistance profiles conferred by chimeric proteins were determined in stably transfected cell clones expressing comparable amounts of wild-type or chimeric P-gps. Immunoblotting experiments showed that all chimeras were found expressed in membrane-enriched fractions of transfected cell clones and all conveyed cellular drug resistance at levels above the background of nontransfected drug-sensitive LR73 cells. For VBL, all chimeric constructs were found to convey similar levels of resistance. For COL and ACT, the levels of resistance conferred by the various chimeras were heterogeneous, being similar to either the parental *mdr1* or the parental *mdr3F* clones, or in many cases being intermediate between the two. The preferential COL and ACT resistance phenotypes of *mdr1* and *mdr3F*, respectively, did not segregate in chimeric proteins with any specific protein segment. Taken together, our results suggest that the preferential drug resistance phenotypes encoded by the *mdr1* and *mdr3F* clones implicate complex interactions between the two homologous halves of the respective P-gp.

Multidrug resistance (MDR)[1] is the phenomenon by which tumor cells in vivo and cultured cells in vitro acquire simultaneous resistance to a large group of structurally and functionally unrelated cytotoxic drugs (Moscow & Cowan, 1988). MDR is caused by the amplification and/or overexpression of a small family of closely related genes termed *mdr* or *pgp*, which code for a group of membrane glycoproteins termed P-glycoproteins (P-gps) (Endicott & Ling, 1989; Roninson, 1991). It has been shown that P-gp is a membrane phosphoglycoprotein capable of combining photoactivatable analogs of ATP (Cornwell et al., 1987; Schurr et al. 1989) and cytotoxic drugs (Cornwell et al., 1986; Safa et al., 1986, 1989) and possesses ATPase activity (Hamada et al., 1988). It is generally accepted that P-gp functions as a drug efflux pump to reduce the intracellular accumulation of cytotoxic drugs in MDR cells. Full-length cDNA clones corresponding to two human *MDR* genes, *MDR1* and *MDR2* (Chen et al., 1986; Van Der Bliek et al., 1987), and three rodent *mdr* genes, *mdr1*, *mdr2*, and *mdr3* (Gros et al., 1986a, 1988; Devault & Gros, 1990; Hsu et al., 1990), have been isolated and sequenced. Analysis of the predicted amino acid sequences of P-gps indicates that they share a high degree of amino acid sequence homology and identical hydropathy profiles. These common structural features include two sequence-homologous halves, each encoding a series of six predicted membrane-associated (TM) domains, and a consensus sequence for nucleotide binding (NB). Each of these homologous segments also shares considerable sequence similarity and possibly common ancestral origin with a group of bacterial genes participating in the extrusion of specific peptide and carbohydrate substrates in Gram-negative bacteria, such as *HlyB* (Felmlee et al., 1985), *LktB* (Stanfield et al., 1988), and *CyaB* (Glaser et al., 1988). The *mdr* gene family has been recently found to be a member of a larger family of sequence-related genes which include the *Saccharomyces cerevisiae* STE6 gene implicated in transport of the **a** mating pheromone (McGrath et al., 1989), the malarial *Plasmodium falciparum pfmdr1* gene associated with chloroquine resistance (Foote et al., 1989), the *CFTR* chloride channel in which mutations cause cystic fibrosis in humans (Riordan et al., 1989), and the *HAM/PSF/RING/mtp* peptide transporters participating in antigen presentation in lymphocytes (Monaco et al., 1990; Spies et al., 1990; Trowsdale et al., 1990; Deverson et al., 1990).

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[1] Abbreviations: VBL, vinblastine; COL, colchicine; ACT, actinomycin D; MDR, multidrug resistance; P-gp, P-glycoprotein; TM, transmembrane; LK, linker; BSA, bovine serum albumin; PCR, polymerase chain reaction; G418, genetycin; DMSO, dimethyl sulfoxide.

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