Specific proteolytic modification of creatine kinase isoenzymes

Implication of C-terminal involvement in enzymic activity but not in subunit–subunit recognition

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We are using the isoenzymes of creatine kinase (CK) to investigate the effect of specific proteolytic modification on the abilities of enzyme subunits to establish precise subunit–subunit recognition *in vitro*. Previous work by others has shown that treatment of the MM isoenzyme of rabbit CK with Proteinase K results in a specific proteolytic modification and inactivation of the enzyme. In the present work, we show that both the MM and BB isoenzymes of chicken CK are also specifically modified by Proteinase K, resulting in over 98% loss of catalytic activity and approx. 10% decreases in subunit molecular masses of the enzymes. Similar reactions appear to occur when the isoenzymes are treated with Pronase E. Limited amino acid sequence analysis of intact and Proteinase K-modified MM-CK suggests that the proteolytic modification results from a single peptide-bond cleavage occurring between alanine residues 328 and 329, about 50 amino acid residues from the C-terminal end; the active-site cysteine residue was recovered in the large protein fragment of modified M-CK subunits. Proteolytically modified M-CK and B-CK subunits were able to refold and reassociate into dimeric structures after treatment with high concentrations of LiCl and at low pH. Thus the proteolytically modified CK subunits retain their ability to refold and to establish precise subunit–subunit recognition *in vitro*.

INTRODUCTION

Much attention has been focused on elucidating the structural and functional characteristics of a variety of proteins. One approach in this endeavour is to modify native protein structure deliberately and to monitor the effect(s) of this modification on the ability of the protein to perform its specific biochemical function(s). A particularly vivid example of the usefulness of this approach is illustrated by the early work of Anfinsen and associates (Anfinsen, 1967) on pancreatic ribonuclease.

In addition to having effects on the catalytic activity of enzymes, specific limited proteolytic modification of oligomeric proteins may alter the ability of protein subunits to establish precise subunit–subunit recognition. We have been using the isoenzymes of CK to investigate the effects of limited proteolysis on the ability of enzyme subunits to refold and reassociate *in vitro*. In avian and mammalian systems, two subunit types of CK, termed M and B, are encoded for by two homologous structural genes (Caplan *et al.*, 1983; Dawson *et al.*, 1968). These subunits can interact, both *in vivo* and *in vitro*, to produce dimeric structures in the forms of MM, MB and BB (Caplan *et al.*, 1983; Dawson *et al.*, 1968).

Several workers have shown that treatment of rabbit MM-CK with the fungal proteinase Proteinase K results in inactivation of the enzyme (Williamson *et al.*, 1977; Price *et al.*, 1981) that is associated with an apparent specific cleavage of the enzyme into a peptide portion containing about 10% of the molecule and a larger

fragment containing the remaining 90%; however, the specific location of this cleavage site has not been previously determined.

The present work shows that chicken MM-CK and BB-CK isoenzymes are inactivated by treatment with Proteinase K and Pronase E and that the enzyme inactivation is associated with approx. 10% decrease in subunit sizes of the enzymes.

Further, the results of our amino acid sequence analysis suggest that the site of cleavage occurs between alanine residues 328 and 329, about 50 residues from the C-terminal end of MM-CK. Finally, we show that the proteolytically modified M-CK and B-CK subunits retain their ability to refold and reassociate into dimeric structures after treatment with high concentrations of LiCl and at low pH.

EXPERIMENTAL

Isolation of MM-CK and BB-CK isoenzymes

MM-CK was isolated from chicken breast muscle as previously described (Petell *et al.*, 1981, 1982). Chicken gizzard contains only BB-CK (Rosenberg *et al.*, 1981), and this isoenzyme was purified as follows. Frozen gizzards (50 g wet wt.) were thawed and fatty tissue was removed. After being minced with scissors, the tissue was homogenized in 10 vol. of 10 mM-Tris/HCl buffer, pH 7.3, containing 1 mM-EDTA, 1 mM-magnesium acetate and 1 mM-2-mercaptoethanol with three 60 s bursts

Abbreviations used: CK, creatine kinase; BB-CK, brain isoenzyme of CK; MM-CK, muscle isoenzyme of CK; MB-CK, hybrid isoenzyme of CK; SDS, sodium dodecyl sulphate; CPM, 7-diethylamino-3-(4-maleimidylphenyl)-4-methylcoumarin.

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in the Sorvall Omnimixer at top speed. The homogenate was centrifuged at 12000 *g* for 10 min, and the supernatant fraction was adjusted to 40% $(\text{NH}_4)_2\text{SO}_4$ saturation by slow addition of the solid salt. After the mixture had been stirred for 30 min, precipitated protein was collected by centrifugation. The precipitate was dissolved in 1 mM-Mes/NaOH buffer, pH 6, containing 1 mM-EDTA and 1 mM-2-mercaptoethanol, and the sample dialysed against the same buffer. After centrifugation to remove any precipitated protein, the sample was applied to a Schleicher and Schuell phosphocellulose column (1.5 cm $\times$ 15 cm) that had been equilibrated in the same buffer. Under these conditions BB-CK is not retained on the column. This material was titrated to pH 8 with 1 M-Tris base and was applied to a Whatman DE-52 DEAE-cellulose column (1.5 cm $\times$ 30 cm) previously equilibrated in 10 mM-Tris buffer, pH 8, containing 1 mM-EDTA and 1 mM-2-mercaptoethanol. The column was resolved with a 0–0.5 M-NaCl gradient (500 ml total) prepared in the above Tris buffer. Peak fractions of CK activity were pooled, and the pool was dialysed against 10 mM-Tris buffer, pH 7.5, containing 1 mM-EDTA and 1 mM-2-mercaptoethanol.

Enzymic activity measurements and protein determinations

CK activity was measured at 25 °C by using the continuous spectrophotometric procedure described by Eppenberger *et al.* (1967). Protein concentrations were determined by the A_{280}/A_{260} method described by Warburg & Christian (1942).

Electrophoretic procedures

Electrophoresis of CK preparations in 9% polyacrylamide slab gels containing 0.1% SDS was performed with the gel reagents and buffer system suggested by Laemmli (1970). Electrophoresis of enzyme samples in non-denaturing 5% polyacrylamide slab gels was performed as previously described (Gabriel, 1971; Lebherz, 1975). All gels were stained for protein with Coomassie Blue. In those cases where the protein, or fragments thereof, was to be isolated from the gel for sequencing, the methods and precautions described by Hunkapiller *et al.* (1983) were used. Recovery of the stained proteins was achieved by electro-elution; the proteins were freed of contaminating SDS, NH_4HCO_3 and Coomassie Blue by ethanol precipitation. Cellulose polyacetate strip electrophoresis followed by staining the strips specifically for CK activity was performed as described by Turner *et al.* (1974).

Proteolytic modification of MM-CK and BB-CK isoenzymes with Proteinase K and Pronase E

Enzyme preparations (approx. 1 mg/ml), equilibrated in 10 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-EDTA and 0.2 mM-2-mercaptoethanol, were incubated in 1.5 ml polypropylene tubes at 37 °C with the appropriate proteinase. Weight ratios of CK to Proteinase K (Merck) were 100:1 and CK to Pronase E (Sigma Chemical Co.) were 20:1. At various times samples were removed for enzyme activity measurements and electrophoretic analysis. Inhibition of Proteinase K activity was achieved by adjusting the solutions to 1 mM-phenylmethanesulphonyl fluoride.

Modification of MM-CK isoenzyme with CPM

A freeze-dried MM-CK preparation was dissolved in 50 mM-sodium phosphate buffer, pH 6.8, at 4 mg/ml. A

Fig. 1. SDS/polyacrylamide-gel-electrophoretic analysis of MM-CK and BB-CK before and after proteolytic modification

Samples of intact MM-CK and BB-CK (lanes 1) were incubated with Proteinase K (lanes 2) or Pronase E (lanes 3) for 90 min at 37 °C as described in the Experimental section. Then samples containing 10–15 μg of protein were subjected to electrophoretic analysis. Molecular-mass markers used were: glyceraldehyde-3-phosphate dehydrogenase, 37 kDa (Hoagland & Teller, 1969); fructose biphosphate aldolase, 40 kDa (Kawahara & Tanford, 1966); M-CK subunit, 43 kDa (Morimoto & Harrington, 1972); enolase, 50 kDa (Gockel & Lebherz, 1981).

2-fold molar excess of CPM over protein thiol groups (Yamamoto *et al.*, 1977) dissolved in acetone was added, and the mixture was incubated on ice for 1 h. The modified protein was isolated from unchanged CPM by chromatography on a Pharmacia PD-10 column equilibrated in 10 mM- NH_4HCO_3 . A portion of the modified MM-CK (approx. 150 μg) was treated with Proteinase K (1 μg) for 1 h at 37 °C, and then the reaction was terminated by addition of 25 mM-phenylmethanesulphonyl fluoride in ethanol to a final concentration of 0.5 mM. Portions of unmodified, modified and digested CK were analysed on a gradient SDS/6–20% polyacrylamide slab gel (Margolis & Kenrick, 1968). The gel was photographed under u.v. illumination on type 667 film (Polaroid), before staining with Coomassie Blue.

N-Terminal sequence analysis of intact and proteolytically modified CK subunits

Intact MM-CK and BB-CK, as well as the MM-CK modified with Proteinase K, were subjected to automated Edman degradation in an Applied Biosystems model 470A gas-phase sequencer. Reagents and solvents for the instrument were supplied by the manufacturer. The phenylthiohydantoin derivatives were identified by reverse-phase h.p.l.c. essentially as described by Johnson *et al.* (1979).

'Hybridization' between intact and proteolytically modified CK subunits

Mixtures of CK, in the molar ratios indicated in the legends of the appropriate Figures, at a final protein concentration of 1 mg/ml were denatured in acid by titration to pH 2.3 as previously described for the dissociation of aldolase tetramers (Heil & Lebherz, 1978). After reassociation and concentration of the samples by ultrafiltration, portions were subjected to both polyacryl-

Fig. 2. SDS/polyacrylamide-gel electrophoresis of CPM-labelled chicken MM-CK before and after proteolysis

Lanes a, MM-CK; lanes b, MM-CK plus Proteinase K. The left-hand panel shows the Coomassie Blue staining pattern, and the right-hand panel shows the fluorescence pattern.

amide and cellulose polyacetate strip electrophoresis as described above.

For dissociation in LiCl, CK mixtures at concentrations of about 1 mg/ml were adjusted to 3 M-LiCl by addition of an equal volume of 6 M-LiCl prepared in 10 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-EDTA and 100 mM-2-mercaptoethanol. After being stirred for 30 min at room temperature, samples were rapidly diluted into 9 vol. of 10 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-EDTA, 50 mM-2-mercaptoethanol and 20% (v/v) glycerol. The solutions were concentrated and subjected to electrophoretic analysis as described above.

Recoveries of CK catalytic activity after the acid and LiCl treatments were usually above 60%.

RESULTS

The MM-CK and BB-CK isoenzymes were isolated as described in the Experimental section. As shown by the results of SDS/polyacrylamide-gel electrophoresis in Fig. 1, intact B-CK subunits (right-hand panel, lane 1) were about 4 kDa larger than the corresponding intact M subunits (left-hand panel, lane 1). Perriard *et al.* (1978) have also noted a considerable size difference between M and B subunits of chicken CK. Also, the report by Perryman *et al.* (1983) suggests that similar differences in molecular mass, i.e. approx. 4 kDa, exists between the B and M subunits isolated from dog, human, rabbit and mouse tissues. Microheterogeneity was often apparent in pure preparations of both chicken isoenzymes (results not shown) and has been observed by Williamson *et al.* (1977) for rabbit MM-CK. George *et al.* (1984) have indicated that multiple forms of CK can arise from proteolysis at the C-terminus of the enzyme, and work by others (Rosenberg *et al.*, 1981, 1982; Schweinfest *et al.*, 1982) has shown that microheterogeneity is already apparent in the primary translation product encoded for by M-CK-subunit mRNA. Thus it appears that there are, minimally, two different routes leading to CK microheterogeneity.

Treatment of the CK isoenzymes with low concentrations of Proteinase K (Fig. 1, lanes 2) resulted in an approx. 4 kDa decrease in the size of M subunits and an approx. 6 kDa decrease in the size of B subunits. Similar results were obtained when the isoenzymes were treated

	1	10
M:	Pro-Phe-Ser-Ser-Thr-His-Asn-Lys-His-Lys-	
B:	Pro-Phe-Ser-Asn-Ser-His-Asn-Leu-Leu-Lys-	
	11	20
M:	Leu-Lys-Phe-Ser-Ala-Glu-Glu-Glu-Phe-Pro-	
B:	Met-Lys-Tyr-Ser-Val-Asp-Asp-Glu-Tyr-Pro-	
	21	30
M:	Asp-Leu-Ser-Lys-His-Asn-Asn-(His)-Met-Ala	
B:	Asp-Leu-Ser- ? -His-Asn-Asn-(His)-Met-Ala	

Fig. 3. N-Terminal amino acid sequences of chicken M-CK and B-CK subunits

Chicken MM-CK and BB-CK were subjected to automated amino acid sequence analysis as described in the Experimental section. M: sequence of MM-CK. B: sequence of BB-CK.

with Pronase E instead (Fig. 1, lanes 3). As may be predicted from the work of others (Williamson *et al.*, 1977; Price *et al.*, 1981) on rabbit MM-CK, the conversion of intact into proteolytically modified chicken CK subunits was associated with a first-order loss of catalytic activity of the enzymes (results not shown). Also, as expected from the work on rabbit CK (Williamson *et al.*, 1977; Price *et al.*, 1981), no intermediates between intact and fully modified CK subunits were detected, suggesting that the proteolytic modifications occur by a single or very few proteolytic 'hits' near one or both ends of CK polypeptide chains.

CK subunits contain a single active-site cysteine residue, which can be modified by reaction with various thiol-blocking reagents, causing loss of enzymic activity. Here we show that this cysteine residue is contained within the large protein fragment of Proteinase K-modified M-CK subunits. MM-CK was labelled with the fluorescent reagent CPM as described by Grossman (1983) and then was digested. Electrophoretic analysis (Fig. 2, right-hand panel) revealed that the modified cysteine residue remained associated with the larger protein fragment of the enzyme.

Amino acid sequence analysis on intact and Proteinase K-modified CK was performed in order to localize the position(s) susceptible to proteolytic modification. The N-terminal amino acid sequences of chicken M-CK and B-CK subunits determined by us are presented in Fig. 3. There was excellent agreement between our amino acid sequence analysis of M-CK subunits and the N-terminal sequence deduced from nucleotide sequence analysis of M-CK cDNA (Ordahl *et al.*, 1984). Further, there was extensive amino acid sequence homology between the N-terminal regions of intact chicken M-CK and B-CK subunits (Fig. 3). These similarities are in keeping with the premise that the basic structure of M-CK and B-CK subunits has been highly conserved during evolution.

In contrast with the single sequence observed for intact M-CK subunits, amino acid sequence analysis of Proteinase K-modified M-CK subunits demonstrated the presence of two polypeptide chains in approximately equal molar amounts. As expected, one sequence was identical with that determined for the intact M subunit. The other sequence is presented in Fig. 4, along with a partial amino acid sequence of M-CK subunit beginning

328 338
 M-CK: Ala-Ala-Val-Gly-Ala-Val-Phe-Asp-Ile-Ser-Asn-
 Fragment: Ala-Val-Gly-Ala-Val-Ser-Asp-Ile-Ser-Asn-
 339 348
 M-CK: Ala-Asp-Arg-Leu-Gly-Phe-Ser-Glu-Val-Glu
 Fragment: Ala-Asp-Arg-Leu-Gly-Phe-Ser-Glu-Val-Glu

Fig. 4. Comparisons between a portion of the amino acid sequence of M-CK subunit and the *N*-terminal sequence of the M-CK-subunit fragment generated by Proteinase K digestion of MM-CK

The *N*-terminal residue of the fragment is aligned with alanine residue 329 of the M-CK subunit primary structure.

with alanine residue 328 deduced from nucleotide sequence data (Ordahl *et al.*, 1984). With the exception of a discrepancy at position 334, an exact correspondence between the *N*-terminal 20 amino acid residues of the peptide fragment and residues 329–348 of the M-CK subunit was observed; thus the Proteinase K modification of M-CK subunits appears to be caused by a single peptide-bond cleavage between alanine residues 328 and 329.

We next investigated the effect of the proteolytic modifications on the ability of CK subunits to refold and reassociate after being denatured *in vitro* by testing the ability of intact and modified subunits to hybridize with each other. As shown in Fig. 5 (top left-hand panel), a three-membered set of activities was produced after treatment of a mixture of intact MM-CK and BB-CK isoenzymes with 3 M-LiCl; the middle activity corresponds to the MB-CK heterodimer. When mixtures of intact

Fig. 5. Detection of CK dimers containing active and Proteinase K-inactivated M-CK and B-CK subunits

Samples of MM-CK and BB-CK were treated with Proteinase K for 90 min. Then the samples were adjusted to 1 mM-phenylmethanesulphonyl fluoride. Mixtures of intact and proteolytically modified enzymes were treated with LiCl or acid. Afterwards, samples were subjected to cellulose polyacetate strip electrophoresis and the strips were stained for CK activity. M' and B' refer to the use of modified subunits. In the LiCl experiments, intact and modified subunits were mixed in molar ratios of 1:3. In the acid experiments the subunit types were mixed in approx. 1:1 molar ratios.

Fig. 6. Hybridization between intact and Proteinase K-modified CK subunits by acid treatment as revealed by polyacrylamide-gel electrophoresis

Intact and Proteinase K-modified MM-CK and BB-CK in about 1:1 molar ratios were treated with acid. After neutralization, the samples were subjected to polyacrylamide-gel electrophoresis under non-denaturing conditions and the gel was stained for protein. Formation of CK dimers containing modified M and intact B (lane 1), intact M and modified B (lane 2), modified M and modified B (lane 3) and intact M and intact B (lane 4) subunits was readily observed.

BB-CK and proteolytically modified MM-CK (Fig. 5, middle left-hand panel) or intact MM-CK and modified BB-CK (Fig. 5, bottom left-hand panel) isoenzymes were treated with LiCl, two-membered sets of activity were generated; note that with this activity stain completely inactive CK dimers would not be detected. Similar results were obtained after acid dissociation of mixtures of these proteins (Fig. 5, right-hand panels). Since the acid treatment probably disrupts tertiary as well as quaternary enzyme structure (Stellwagen & Schachman, 1962), these data suggest that proteolytically modified CK polypeptide chains are able to refold into an appropriate three-dimensional conformation so that they can reassociate with other subunits.

Proteolytically modified CK subunits can also associate with each other, as revealed by electrophoresis of 'hybrid' sets in 5% polyacrylamide slab gels under non-denaturing conditions followed by staining the gels for protein. Three-membered sets of proteins were detected in hybridization experiments with intact and proteolytically modified MM-CK and BB-CK in all possible combinations (Fig. 6).

DISCUSSION

Treatment of both MM and BB isoenzymes of chicken CK with Proteinase K and Pronase E resulted in essentially complete inactivation of the enzymes with approx. 10% decreases in subunit molecular masses of these proteins. Results of our limited amino acid sequence analysis strongly suggest that the Proteinase K modification results from a single peptide-bond cleavage

occurring between alanine residues 328 and 329. Because of the extensive homology that exists between M-CK and B-CK subunits (Fig. 3) it is probable that a structurally homologous proteolytic cleavage site is present in the C-terminal region of B-CK subunits.

It was previously shown (Price *et al.*, 1981) that Proteinase K modification of rabbit MM-CK resulted in altered susceptibility of the active-site cysteine residue of the enzyme. The present work directly demonstrates that this cysteine residue is located within the large protein fragment of proteolytically modified M-CK subunits (see Fig. 2). This observation is consistent with the established primary structure of chicken M-CK subunits, which locates this cysteine at position 283 of M-CK subunits (Ordahl *et al.*, 1984), some 45 amino acid residues 'upstream' from the Proteinase K-cleavage site. It may also be noted that other amino acid residues believed to be involved in the catalytic function of CK are also to the N-terminal side (Ordahl *et al.*, 1984; Benfield *et al.*, 1984; Putney *et al.*, 1984) of the Proteinase K-cleavage site described here. It is not yet known whether additional amino acid residues located in the small peptide fragment of modified M-CK subunits also participate directly in the catalytic cycle of the enzyme.

The proteolytic cleavage of CK described here is also of interest in terms of the substrate specificities assigned to Pronase E and Proteinase K. Pronase E is a rather heterogeneous mixture of proteinases (Seber *et al.*, 1976) and contains two rather well-characterized aminopeptidases (Vosbeck *et al.*, 1975). These exopeptidases do not cleave N-terminal proline residues (Vosbeck *et al.*, 1975). Since intact M-CK and B-CK subunits have proline at the N-terminus, it is not surprising that these amino peptidases do not remove these residues from CK subunits. It is possible that the rather impure Pronase preparations used here contain an endopeptidase (Seber *et al.*, 1976), the specificity of which may be similar to that expressed by Proteinase K. The electrophoretically homogeneous preparations of Proteinase K (a serine proteinase) used here have been shown to exhibit preference for carboxy groups adjacent to aliphatic and aromatic amino acids, in particular to alanine residues (Ebeling *et al.*, 1974). Thus the cleavage of a peptide bond between alanine residues 328 and 329 of MM-CK by this proteinase is not surprising. What is surprising is that Proteinase K digestion of CK molecules is so limited. Proteinase K is a powerful proteolytic enzyme with a fairly broad substrate specificity. In fact, this proteinase is commonly used to digest unwanted protein during the isolation of nucleic acids (Wieggers & Hilz, 1971; Gross-Bellard *et al.*, 1973). Thus there must be a specific region of CK subunits that is particularly susceptible to attack by this proteinase, while numerous other regions of these molecules are relatively resistant to attack.

Finally, the present work shows that the proteolytically modified M-CK and B-CK subunits retain their abilities to refold and reassociate into dimeric structures after treatment with high concentrations of LiCl or at low pH. Thus CK subunits that have been proteolytically cleaved about 50 amino acid residues away from their C-terminal ends retain their ability to refold and to establish precise subunit-subunit recognition *in vitro*.

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REFERENCES

- Anfinsen, C. B. (1967) Harvey Lect. **61**, 95–116
- Benfield, P. A., Zivin, R. A., Miller, L. S., Sowder, R., Smythers, G. W., Henderson, L., Oroszlan, S. & Pearson, M. L. (1984) *J. Biol. Chem.* **259**, 14979–14984
- Caplan, A. I., Fiszman, M. Y. & Eppenberger, H. M. (1983) *Science* **221**, 921–924
- Dawson, D. M., Eppenberger, H. M. & Kaplan, N. O. (1968) *Ann. N.Y. Acad. Sci.* **151**, 616–626
- Ebeling, W., Hennrich, N., Klockow, M., Metz, H., Orth, H. D. & Lang, H. (1974) *Eur. J. Biochem.* **47**, 91–97
- Eppenberger, H. M., Dawson, D. M. & Kaplan, N. O. (1967) *J. Biol. Chem.* **242**, 204–209
- Gabriel, O. (1971) *Methods Enzymol.* **22**, 565–578
- George, S., Ishikawa, Y., Perryman, M. B. & Roberts, R. (1984) *J. Biol. Chem.* **259**, 2667–2674
- Gockel, S. F. & Lebherz, H. G. (1981) *J. Biol. Chem.* **256**, 3877–3882
- Gross-Bellard, M., Oudet, P. & Chambone, P. (1973) *Eur. J. Biochem.* **36**, 32–38
- Grossman, S. H. (1983) *Biochemistry* **22**, 5369–5375
- Heil, J. A. & Lebherz, H. G. (1978) *J. Biol. Chem.* **253**, 6599–6605
- Hoagland, V. D. & Teller, D. C. (1969) *Biochemistry* **8**, 594–602
- Hunkapiller, M. W., Lujan, E., Ostrander, R. & Hood, L. E. (1983) *Methods Enzymol.* **91**, 227–236
- Johnson, N. D., Hunkapiller, M. W. & Hood, L. E. (1979) *Anal. Biochem.* **110**, 335–338
- Kawahara, K. & Tanford, C. (1966) *Biochemistry* **5**, 1578–1584
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lebherz, H. G. (1975) *J. Biol. Chem.* **250**, 5976–5981
- Margolis, J. & Kenrick, K. G. (1968) *Anal. Biochem.* **25**, 347–362
- Morimoto, K. & Harrington, W. F. (1972) *J. Biol. Chem.* **247**, 3052–3061
- Ordahl, C. P., Evens, G. L., Cooper, T. A., Kunz, G. & Perriard, J. C. (1984) *J. Biol. Chem.* **259**, 15224–15227
- Perriard, J. C., Perriard, E. R. & Eppenberger, H. M. (1978) *J. Biol. Chem.* **253**, 6529–6535
- Perryman, M. B., Strauss, A. W., Buettner, T. L. & Roberts, R. (1983) *Biochim. Biophys. Acta* **747**, 284–290
- Petell, J. K., Sardo, M. J. & Lebherz, H. G. (1981) *Prep. Biochem.* **11**, 69–89
- Petell, J. K., Killion, R. & Lebherz, H. G. (1982) *Methods Enzymol.* **90**, 490–497
- Price, N. C., Murray, S. & Milner-White, E. J. (1981) *Biochem. J.* **199**, 239–244
- Putney, S., Herlihy, W., Royal, N., Pang, H., Aposhian, H. V., Pickering, L., Belagaje, R., Biemann, K., Page, D., Kuby, S. & Schimmel, P. (1984) *J. Biol. Chem.* **259**, 14317–14320
- Rosenberg, U. B., Eppenberger, H. M. & Perriard, J. C. (1981) *Eur. J. Biochem.* **116**, 87–92
- Rosenberg, U. B., Kunz, G., Frischauf, A., Lehrach, H., Mahr, R., Eppenberger, H. M. & Perriard, J. C. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6589–6592
- Schweinfest, C. W., Kwiatkowski, R. W. & Dottin, R. P. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4997–5000
- Seber, J. F., Toomey, T. P., Powell, J. T., Brew, K. & Awad, W. M., Jr. (1976) *J. Biol. Chem.* **251**, 204–208
- Stellwagen, E. & Schachman, H. K. (1962) *Biochemistry* **1**, 1056–1069
- Turner, D. C., Maier, V. & Eppenberger, H. M. (1974) *Dev. Biol.* **37**, 63–89
- Vosbeck, K. D., Greenberg, B. D. & Awad, W. M., Jr. (1975) *J. Biol. Chem.* **250**, 3981–3987

- Warburg, O. & Christian, W. (1984) *Biochem. Z.* **310**, 384–421
- Wieggers, U. & Hilz, H. (1971) *Biochem. Biophys. Res. Commun.* **44**, 513–519

- Williamson, J., Greene, J., Cherif, S. & Milner-White, E. J. (1977) *Biochem. J.* **167**, 731–737
- Yamamoto, K., Sekine, T. & Kanaoka, Y. (1977) *Anal. Biochem.* **79**, 83–94

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