Rotilio, G., Bray, R. C., & Fielden, E. M. (1972) *Biochim. Biophys. Acta 268*, 605–609.
Rousch, A., & Norris, E. R. (1950) *Arch. Biochem. Biophys. 29*, 344–347.
Steinman, H. M., & Hill, R. L. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3725–3729.
Steinman, H. M., Naik, V. R., Abernethy, J. L., & Hill, R. L. (1974) *J. Biol. Chem. 249*, 7326–7338.
Takahashi, K. (1968) *J. Biol. Chem. 243*, 6171–6179.
Weisiger, R. A., & Fridovich, I. (1973) *J. Biol. Chem. 248*, 3582–3592.
Weng, L., Heinrikson, R. L., & Westley, J. (1978) *J. Biol. Chem. 253*, 8109–8119.
Yang, P. C., & Schwert, G. W. (1972) *Biochemistry 11*, 2218–2224.
Yankeelov, J. A. (1970) *Biochemistry 9*, 2433–2439.
Yost, F. J., & Fridovich, I. (1973) *J. Biol. Chem. 248*, 4905–4908.

Subunit-Selective Chemical Modifications of Creatine Kinase. Evidence for Asymmetrical Association of the Subunits†

Yair Degani* and Chemda Degani*

ABSTRACT: The two reactive thiol groups in the dimeric enzyme creatine kinase (CK) react nonidentically with the cyanylating reagent 2-nitro-5-thiocyanobenzoic acid (NTCB). While in one subunit the thiol undergoes cyanylation, the other subunit thiol abnormally forms a mercaptonitrobenzoate (TNB) mixed disulfide. The resulting derivative, *S*-CN-*S'*-TNB-CK, is catalytically inactive. Cyanolysis of this derivative with KCN rapidly produces the dicyano enzyme *S,S'*-di-CN-CK, which possesses 75% of the original enzymic activity. The same active derivative is also formed by total cyanolysis of the inactive derivative *S,S'*-di-TNB-CK, produced by a previous reaction of the enzyme with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). During the cyanolysis of *S,S'*-di-TNB-CK, one TNB group is displaced much more rapidly than the other. The regeneration of enzymic activity coincides solely with the faster of the two reactions. The 75%-active half-cyanolysis product, *S*-TNB-*S'*-CN-CK, is the isomer of the inactive *S*-CN-*S'*-TNB-CK produced by NTCB. These results suggest that the subunits of CK are asymmetrically associated. Difference spectrophotometry measurements have shown that the inactive derivative *S*-CN-*S'*-TNB-CK is capable of forming the ternary complex E·MgADP·creatine at both of its subunits but fails to form the quaternary transition-state analogue (t.s.a.) complex E·MgADP·NO_3^-·creatine at either subunit. In contrast, the 75% active *S,S'*-di-CN-CK is capable of forming the t.s.a. complex at each of the subunits. Hence, the single TNB blocking group in *S*-CN-*S'*-TNB-CK which prevents catalysis in the neighboring cyanylated subunit also eliminates the ability of that subunit to form the t.s.a. complex.

In almost all of the oligomeric proteins whose quaternary structure has been determined by X-ray crystallography, the protomers were found to be arranged in a strictly symmetrical manner, as had been postulated by Monod et al. (1965). In three exceptional cases, insulin (Adams et al., 1969), α-chymotrypsin (Birktoft & Blow, 1972; Tulinsky et al., 1973), and yeast hexokinase B (Steitz et al., 1973; Anderson et al., 1974; Anderson & Steitz, 1975), asymmetry in the apoprotein arrangement in the crystal has been observed. However, the relevance of these crystallographic observations to the state of these proteins in solution has been questioned (Levitzki & Koshland, 1976; Hoggett & Kellett, 1976). Thus far, there has been no evidence to show that identical protomers can combine in an asymmetrical manner in solution phase.

In this paper we present results of chemical modification studies on the dimeric enzyme creatine kinase, suggesting that the subunits in this enzyme are associated asymmetrically in solution. The enzyme (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) contains two catalytic sites and two reactive thiol groups per dimer of molecular weight 82 600 [for a review, see Watts (1973)]. In denaturating media the enzyme dissociates into two subunits, each consisting of a single polypeptide chain. Detailed analyses of the tryptic peptides, coupled with quantitative end group analyses (Yue et al., 1967) as well as peptide mapping in a variety of different systems (Dance & Watts, 1962; Thomson et al., 1968; Gosselin-Rey & Gerady, 1970; Kumudavalli et al., 1970; Atherton & Thomson, 1969; Focant, 1970), point heavily to an identity of the two polypeptide chains. In particular, the two reactive thiol groups were shown to be identical, in that they both formed part of a unique 25-residue tryptic peptide sequence (Thomson et al., 1964; Atherton et al., 1970; Mahowald, 1965). In this paper we report that these two sequentially identical thiol groups react in a nonidentical manner with the cyanylating reagent 2-nitro-5-thiocyanobenzoic acid to form a catalytically inactive enzyme derivative, differentially modified at its two subunits. By demonstrating the formation of the highly active isomer of this derivative, we provide evidence to suggest that the subunits in this enzyme are asymmetrically associated.

Materials and Methods

Materials. Rabbit muscle creatine kinase (CK)[1] was obtained from Sigma. Most of the work described herein was done with enzyme from lot 115C-9580. The enzyme was found homogeneous in the following systems: (1) electrophoresis on 7% polyacrylamide gel at pH 8.3 (Davis, 1964);

† From the Departments of Organic Chemistry (Y.D.) and Isotope Research (C.D.), The Weizmann Institute of Science, Rehovot, Israel. *Received February 23, 1978; revised manuscript received September 12, 1979.* This work was supported in part by Grant No. AM-5098 from the U.S. Public Health Service.

[1] Abbreviations used: CK, creatine kinase; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); NTCB, 2-nitro-5-thiocyanobenzoic acid; TNB, 2-mercapto-5-nitrobenzoic acid or its residue in mixed disulfide linkage; t.s.a., transition-state analogue.

0006-2960/79/0418-5917$01.00/0

(2) cellulose acetate electrophoresis at pH 8.6 (Turner et al., 1974); (3) electrophoresis on 4% polyacrylamide gel in the presence of 8 M cyanate-free urea at pH 8.3 (Reisfeld & Small, 1966); (4) electrophoresis on 10% polyacrylamide gel in the presence of sodium dodecyl sulfate at pH 8.3 (Laemmli, 1970) (observed molecular weight 41 000, with the following proteins as standards: bovine serum albumin, ovalbumin, deoxyribonuclease, chymotrypsin). The enzyme also appeared homogeneous on sedimentation equilibrium analysis in 0.1 M Tris–acetate buffer at pH 7.0 and 20 °C. From the analysis, a molecular weight of 81 300 ± 1400 was computed by using the value $\bar{V} = 0.745$ cm^3/g, this being the partial specific volume of the native enzyme extrapolated from the data of Noda et al. (1954) to zero concentration at 20 °C (Kuby & Noltmann, 1962). These tests indicated that the enzyme was a pure homodimer. Its specific activity was 120–140 units/mg under the assay conditions indicated below.

Beef heart creatine kinase, rabbit brain creatine kinase, ATP, ADP, and creatine were also from Sigma. DTNB was purchased from Aldrich. Ultrapure guanidinium chloride was from Schwarz/Mann. Potassium [^{14}C]cyanide (53 mCi/mmol) was the product of New England Nuclear. Prior to their use in cyanolysis reactions, fresh solutions of [^{14}C]KCN were ultrafiltered through a UMO5 Diaflo membrane to remove traces of radioactive polymeric contaminants. NTCB was prepared as its potassium half-salt as previously described (Degani & Patchornik, 1971). [^{14}C]NTCB (2.1×10^6 cpm/μmol) was prepared by using [^{14}C]KCN by the same method, scaled down 1000-fold. S-Carboxymethylated creatine kinase was prepared essentially as described by Mahowald et al. (1962).

Assays. Measurements of CK activity were carried out on a Radiometer pH stat assembly (titrigraph SBR2C, titrator TTT11) at pH 8.8 and 30 °C according to the method of Mahowald et al. (1962), except that magnesium acetate was used instead of $MgSO_4$. Protein concentrations were determined from the absorbance at 280 nm, assuming an extinction coefficient of 0.88 mL/(mg cm) (Noda et al., 1954) and a molecular weight of 82000 (Yue et al., 1967). TNB was determined spectrophotometrically at 412 nm by using the molar extinction coefficient of 13 600 (Ellman, 1959; Degani & Patchornik, 1971). NTCB was determined by its quantitative conversion to TNB with an excess of β-mercaptoethanol at pH 7 (Degani & Patchornik, 1971).

Enzyme Modification. All buffers used contained 0.25 mM EDTA. Modifications with DTNB, NTCB, or [^{14}C]NTCB were carried out with enzyme concentrations ranging from 0.02 to 0.2 mM in 0.02 Tris–acetate at pH 7.8 and with 2.5–10 molar excess of reagent. Modified enzyme was separated from excess reagent by Sephadex G-25 column chromatography using Tris–acetate buffer at pH 7.0 as eluant or by dialysis against the same buffer. Protein solutions were concentrated by using Amicon ultrafiltration standard cells with PM-10 membranes. Buffer-change equilibrations of protein solutions were carried out similarly.

Cyanolysis of DTNB-Modified Enzyme. To 1.0 mL of 0.071 mM DTNB-modified enzyme (in 0.065 M potassium phosphate buffer at pH 7.2) was added 0.6 mL of 0.29 M KCN (dissolved in 0.225 M potassium phosphate buffer of pH 7.2); the final pH was 9.5. TNB release and regeneration of enzymic activity were monitored simultaneously. At measured time intervals, 6-μL aliquots were taken from part of the reaction mixture and quenched into an ice-cold 2.4-mL solution of 0.01 M magnesium acetate at pH 6.8. These solutions were subsequently assayed for enzymic activity. Cyanolysis by [^{14}C]KCN (1.94×10^6 cpm/μmol) was carried out under identical conditions. At the end of this reaction, the mixture was diluted 25-fold with cold 0.05 M phosphate buffer at pH 7.0, washed by ultrafiltration, concentrated to a volume of 4 mL, and then exhaustively dialyzed against the same buffer. Cyanolysis of NTCB-modified enzyme by [^{14}C]KCN was carried out by the same method.

Half-Cyanolysis of DTNB-Modified Enzyme. To 2.5 mL of 0.084 mM DTNB-modified enzyme (in 0.05 M potassium phosphate buffer at pH 7.0) was added 1.5 mL of 0.14 M [^{14}C]KCN (1.62×10^6 cpm/μmol; dissolved in 0.5 M potassium buffer of pH 7.0), resulting in a final pH of 8.0. When the absorbance at 412 nm had reached a value corresponding to the release of 0.8 mol of TNB per mol of enzyme, 8 mL of cold saturated ammonium sulfate solution was added to half of the reaction mixture (sample 1) while the other half was quenched into 600 mL of ice-cold 0.01 M phosphate buffer at pH 6.8 (sample 2). Protein precipitated from sample 1 was collected by centrifugation, redissolved in 5 mL of 0.05 M phosphate buffer at pH 8.6, and then dialyzed against the same buffer. Sample 2 was repeatedly washed and concentrated to a volume of 10 mL by ultrafiltrations and then dialyzed against the same buffer.

Other Methods. Sedimentation equilibrium analyses were carried out with a Beckman Model E analytical ultracentrifuge equipped with a split-beam photoelectric scanning absorption system at 10 000 rpm.

Absorbancies were measured on a Zeiss PMQ II spectrophotometer or a Gilford 250 recording spectrophotometer. Absorption spectra were recorded on a Cary 14 spectrometer. Difference spectra measurements were made with a Cary 118 spectrophotometer at 25 °C in split path length cells, the path length of each half-cell being 4.37 mm.

Radioactivity was measured in Bray's solution by using a Packard 3385 liquid scintillation counter.

Results

Reactions with DTNB. Treatment of the enzyme with a 4–10 molar excess of DTNB at pH 7.8 released 1.80 ± 0.05 mol of TNB per mol of enzyme, with consequent loss (over 99.5%) of the enzymic activity. When the modified enzyme was separated from excess reagent, it was found to contain 1.75 ± 0.05 bound TNB groups/mol, as determined by their release on treatment with 0.14 M β-mercaptoethanol. When the enzyme was treated at pH 7.8 with a 30 molar excess of DTNB in the presence of 8 M urea or 6 M guanidinium chloride, 6.3 and 7.2 mol of TNB per mol of enzyme were released, respectively. The aforementioned results are in agreement with previous reports [e.g., Bayley & Thomson (1967), Brown & Cunningham (1970), and O'Sullivan (1971)].

The DTNB-modified enzyme, henceforth referred to as *S,S'*-di-TNB-CK, was unreactive to NTCB.

Reactions with NTCB. The thiol groups of peptides and proteins can be converted quantitatively to their *S*-cyano derivatives by the cyanylating reagent NTCB, with release of TNB (Degani et al., 1970; Degani & Patchornik, 1974; Stark, 1977). When native CK (0.02–0.2 mM) was treated with a 2.5–10 molar excess of NTCB in Tris–acetate buffer, pH 7.8, only 0.73–0.81 mol of TNB per mol of enzyme was released. At the end of the reaction (5–10 min) the enzyme was essentially inactive (retaining less than 0.5% of its original activity). There was no further release of TNB on subsequent treatment with a 10-fold molar excess of DTNB, indicating the absence of any residual reactive thiol groups. The same results were obtained when the enzyme was treated with 1.4

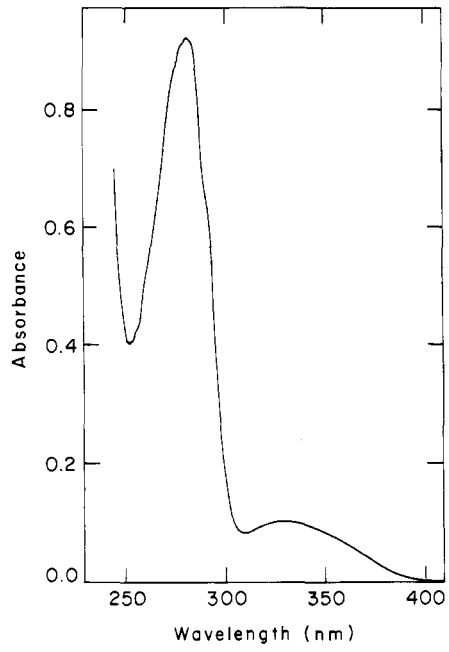

FIGURE 1: Absorption spectrum of NTCB-modified enzyme in 0.02 M Tris–acetate buffer at pH 7.0.

mM β-mercaptoethanol and exhaustively dialyzed against Tris–acetate buffer, pH 7, prior to NTCB modification. When the enzyme was treated with a 2.5–5 molar excess of [^{14}C]-NTCB, followed by gel chromatography, the specific radioactivity of the isolated protein corresponded to incorporation of 0.7–0.8 [^{14}C]cyano group per mol, in accord with the measured release of TNB during the reaction.

The absorption spectrum of the NTCB-modified enzyme (Figure 1) contained a band at 330 nm, characteristic of the TNB mixed disulfide derivative of the enzyme (O'Sullivan, 1971). From the spectrum, a value of 1.06 mol of *S*-TNB groups per mol of enzyme was calculated by using the value ϵ_{330} = 7500 (Price, 1976). Treatment of the NTCB-modified enzyme with 0.14 M β-mercaptoethanol in Tris–acetate buffer at pH 7 released 0.98–1.05 mol of TNB per mol enzyme, thereby restoring the original enzymic activity. In the case of the [^{14}C]NTCB-modified enzyme, the β-mercaptoethanol treatment also released the bound radioactivity, as determined after subsequent dialysis. These results indicated that the NTCB-modified enzyme contained an *S*-cyano group, as well as an *S*-TNB mixed disulfide group. This inactive derivative is designated *S*-CN-*S'*-TNB-CK (I). While the present study was underway, Price (1976) has also described the abnormal reaction of CK with NTCB, although the reported ratio between the two modes of reaction of NTCB differed somewhat from the ratio found by us.

In contrast to the behavior of the native enzyme, the denatured protein reacted normally with NTCB, undergoing essentially quantitative S-cyanylation. Treatment of the enzyme with a 30-fold molar excess of NTCB in the presence of 8 M urea or 6 M guanidinium chloride at pH 7.8 released 5.8 and 6.8 mol of TNB per mol of enzyme, respectively. These values correspond to yields of 92–94% on the basis of the reactions with DTNB under identical conditions. The abnormal reaction of NTCB with native CK appeared, therefore, to result from a specific chemical environment existing at one thiol group in the native enzyme, which promoted S-arylthiolation in place of S-cyanylation. This conclusion was later confirmed by an analogous (though not identical) abnormal reaction of the enzyme with another S-cyanylating reagent, 2,4-dinitrophenyl thiocyanate, to form a catalytically

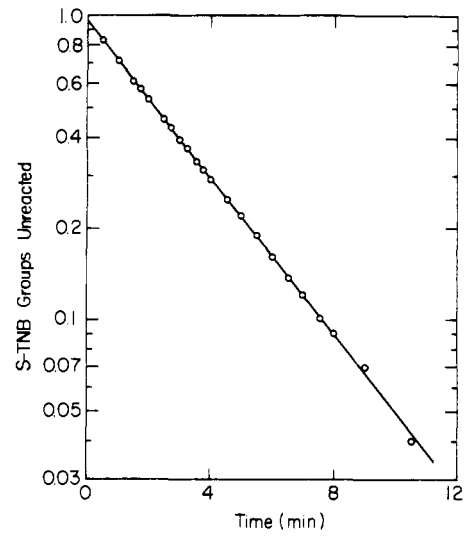

FIGURE 2: Cyanolysis of NTCB-modified enzyme. Semilog plot of the rate of release of TNB on treatment of the modified enzyme with 0.11 M [^{14}C]KCN in 0.125 M potassium phosphate buffer at pH 9.5.

inactive monocyano monodinitrophenyl derivative of the enzyme (C. Degani and Y. Degani, unpublished experiments).

Cyanolysis of S-CN-S'-TNB-CK (I). TNB-cysteine mixed disulfides are known to undergo a cyanolysis reaction with cyanide ion, resulting in selective displacement of TNB and conversion of the cysteine residue into its *S*-cyano derivative (Vanaman & Stark, 1970; Degani et al., 1970). When non-radioactive NTCB-modified enzyme was treated with 0.11 M [^{14}C]KCN in 0.125 M potassium phosphate buffer at pH 9.5, 0.98 mol of TNB per mol of enzyme was released. The reaction followed pseudo-first-order kinetics (Figure 2), with a second-order rate constant of 3.0 M^{-1} min^{-1}. This reaction regenerated 75% of the original enzymic activity. After isolation of the enzyme by ultrafiltration, followed by exhaustive dialysis, the 75%-active derivative was found to possess 1.0 [^{14}C]cyano group/mol by its specific radioactivity. These results confirmed the presence of one *S*-TNB mixed disulfide group in the NTCB-modified enzyme and also showed that the dicyano enzyme (henceforth referred to as *S,S'*-di-CN-CK) possesses 75% of the original enzymic activity. This result is in excellent agreement with the value of 73% residual activity reported by Der Terrossian & Kassab (1976), who prepared the same derivative by total cyanolysis of the DTNB-modified enzyme. A kinetic study of this reaction is presented below.

Cyanolysis of S,S'-Di-TNB-CK. When *S,S'*-di-TNB-CK (containing 1.76 bound TNB groups/mol) was treated with 0.11 M KCN in 0.125 M phosphate buffer at pH 9.5, the rates of TNB release and enzyme reactivation were followed simultaneously. As shown in Figure 3, the bound TNB groups were released quantitatively within 90 min, but the rate of release was clearly biphasic. Strikingly, a maximal value of 75% reactivation was attained within 5 min and remained constant through the rest of the reaction.[2] Figure 4 presents

[2] This constancy depended on the experimental conditions. Having carried out numerous experiments under a variety of conditions, including different buffers, cyanide concentrations, and pH values, as well as different enzyme preparations from various commercial sources, we often observed a gradual decline of the enzymic activity down to a value of 40–50%, particularly under conditions of lower cyanide concentrations and lower pH, which greatly slowed the second phase of the reaction. Nevertheless, in *all* cases, the same maximal value of 70–75% reactivation was invariably attained at a strikingly early stage of the reaction, concurrent with a rapid phase of TNB release. Difficulties in securing the highly active dicyano end product (by this reaction) have also been reported by Smith et al. (1975) and by Der Terrossian & Kassab (1976).

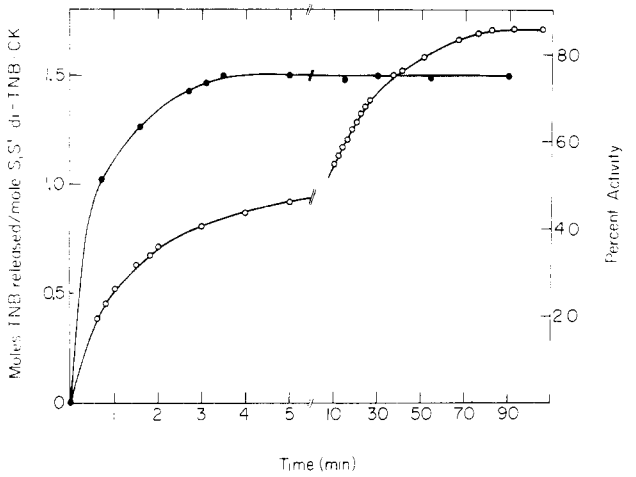

FIGURE 3: Cyanolysis of *S*,*S*′-di-TNB-CK. The modified enzyme was treated with 0.11 M KCN in 0.125 M potassium phosphate buffer at pH 9.5. The reaction was followed by the release of TNB (○) and by the regeneration of enzymic activity (●).

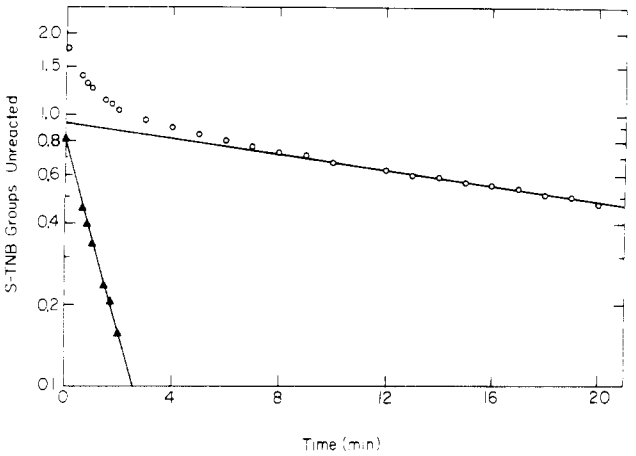

FIGURE 4: Semilog plot for the rate of TNB release during cyanolysis of *S*,*S*′-di-TNB-CK. (○) Experimental data points; (▲) points obtained by subtracting the contribution of the slow reaction from the observed data.

the semilog pseudo-first-order rate plot for the TNB release. The plot is characteristic of two parallel reactions producing a common product (Frost & Pearson, 1961) and was thus analyzed by the method of Brown & Fletcher (1949). The analysis showed that 0.85 ± 0.5 *S*-TNB group/mol of enzyme was involved in the fast phase of the reaction ($t_{1/2}$ = 0.8 min) and 0.90 ± 0.05 *S*-TNB group/mol in the slow phase ($t_{1/2}$ = 21 min). The second-order rate constants for the two parallel reactions were calculated to be 7.8 and 0.3 M^{-1} min^{-1}. Figure 5 shows the semilog plot for the rate of enzyme reactivation. The rate of reactivation followed pseudo-first-order kinetics with a half-life of ~0.6 min, in reasonable agreement with that of the fast TNB release. The results demonstrated that reactivation of the enzyme was directly related to the faster of the two parallel cyanolysis reactions and not with the slower reaction, which did not contribute further to the maximal activity of 75%, possessed by the half-cyanolysis product of *S*,*S*′-di-TNB-CK. This 75%-active monocyanomono-TNB derivative of the enzyme is designated *S*-TNB-*S*′-CN-CK (II) to distinguish it from its inactive isomer *S*-CN-*S*′-TNB-CK (I) produced by NTCB. A summary of the reactions described above is presented in Figure 6.

For verification of the extent of cyano group incorporation during total cyanolysis, the reaction was repeated with [^{14}C]-KCN under identical conditions. At the end of the reaction,

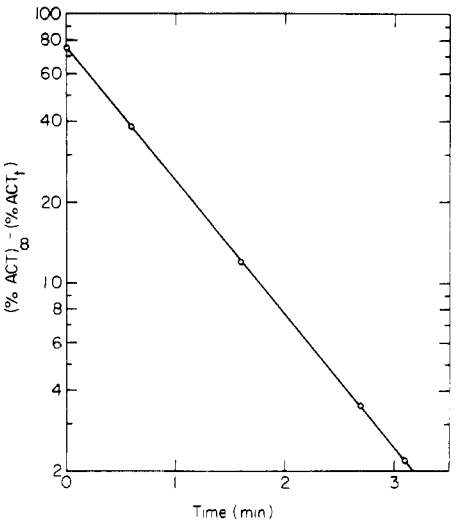

FIGURE 5: Semilog plot for the rate of regeneration of enzymic activity during cyanolysis of *S*,*S*′-di-TNB-CK as described in Figure 3.

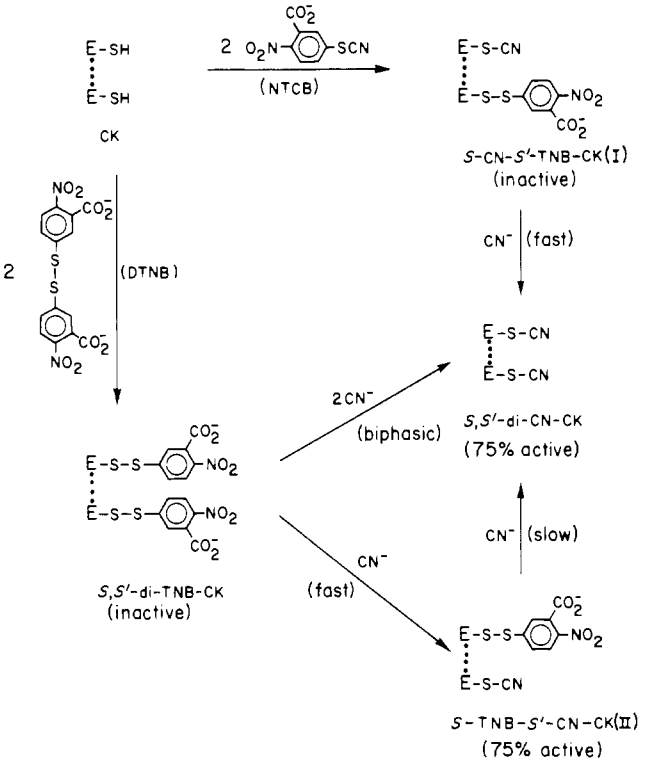

FIGURE 6: Derivatizations of creatine kinase. "E" designates enzyme subunit.

the 75%-active enzyme was separated from the reaction mixture by ultrafiltration and exhaustive dialysis. The specific radioactivity of the isolated enzyme derivative corresponded to incorporation of 1.6 [^{14}C]cyano groups/mol (92% yield with respect to the measured TNB release during the reaction). The isolated enzyme was 70% active and contained no residual *S*-TNB groups, as determined by β-mercaptoethanol. This treatment increased the enzymic activity to its original value. The extent of nonspecific incorporation of ^{14}C label during total cyanolysis was less than 3%, as determined by a parallel control experiment with the S,S′-dicarboxymethylated enzyme under identical conditions.

The large difference between the rate constants of the two parallel reactions occurring during the cyanolysis of *S*,*S*′-di-TNB-CK (Figure 4) prompted an attempt to isolate its half-cyanolysis product. To this end, we moderated the fast

phase of the reaction by employing a lower cyanide concentration at a lower pH. Accordingly, *S,S'*-di-TNB-CK was treated with 0.053 M [^{14}C]KCN in 0.22 M phosphate buffer at pH 8.0, and the reaction was followed spectrophotometrically at 412 nm. When the extent of TNB release reached a value of 0.8 mol/mol of enzyme, the reaction was interrupted and the enzyme recovered. This was achieved by two methods employed simultaneously on two separate samples of the reaction mixture. Sample 1: the enzyme was precipitated with 80% saturated ammonium sulfate, followed by centrifugation and dialysis. Sample 2: the reaction mixture was quenched into 300 volumes of ice-cold buffer at pH 6.8, followed by ultrafiltrations and dialysis. As had been determined, the activity of the enzyme in the reaction mixture just prior to halting the cyanolysis was 70%. Enzyme recovered from sample 1 was 40% active and possessed 0.8 [^{14}C]cyano group/mol of enzyme (by its specific radioactivity) and 1.0 mol of bound TNB (by the absorbance at 412 nm, resulting from β-mercaptoethanol treatment). The partial loss of activity appeared to result from partial denaturation occurring during the rapid ammonium sulfate precipitation in view of separate control experiments with the native enzyme under identical precipitation conditions. Enzyme recovered from sample 2 was 75% active and possessed 0.9 [^{14}C]cyano group and 0.9 *S*-TNB group per mol. These results confirmed the identity of the half-cyanolysis product of *S,S'*-di-TNB-CK as a mono-TNB derivative of the enzyme. Unlike the inactive isomer *S*-CN-*S'*-TNB-CK (I) and the active *S,S'*-di-CN-CK, the active *S*-TNB-*S'*-CN-CK (II) was found to be unstable. On storage, and sometimes even during isolation, this derivative tended to lose part of its TNB content and some of its enzymic activity to a variable extent (see also footnote 2).

Difference Spectra Measurements. The addition of MgADP plus creatine to the 75%-active *S,S'*-di-CN-CK (obtained by cyanolysis of the inactive *S*-CN-*S'*-TNB-CK) produced the difference spectrum shown in Figure 7b-2. Upon subsequent addition of nitrate ions, the features of the difference spectrum became markedly intensified (Figure 7b-3), indicating the formation of the quaternary complex E·MgADP·NO_3^-· creatine. These spectra are essentially identical with those produced by the native enzyme under the same conditions (Figure 7a) and are also identical with those described by Focant & Watts (1973) for the native enzyme under very similar conditions. These results confirm the report of Der Terrossian & Kassab (1976) on the ligand-binding properties of the dicyano enzyme produced by cyanolysis of the DTNB-modified enzyme. Figure 7c presents the spectral behavior of the inactive *S*-CN-*S'*-TNB-CK toward the same ligands. The addition of MgADP plus creatine produced the characteristic difference spectrum for the ternary complex E·MgADP·creatine. However, subsequent addition of nitrate ions had no effect on the difference spectrum, indicating complete failure to form the quaternary E·MgADP·NO_3^-· creatine complex.[3]

Experiments with Creatine Kinase from Other Biological Sources. All of the experiments described above for rabbit muscle CK have also been carried out on beef heart CK (essentially an MM enzyme) with similar results. Experiments on rabbit brain CK (NTCB modification to form the catalytically inactive *S*-CN-*S'*-TNB-CK; rapid cyanolysis of this derivative resulting in regeneration of 70–75% of the original enzymic activity; cyanolysis of the inactive *S,S'*-di-TNB-CK exhibiting kinetic biphasicity of TNB release, coupled with a burst of 70–75% regeneration of maximal enzymic activity) have indicated that the brain isozyme possesses the same anomalous properties as the muscle creatine kinases.

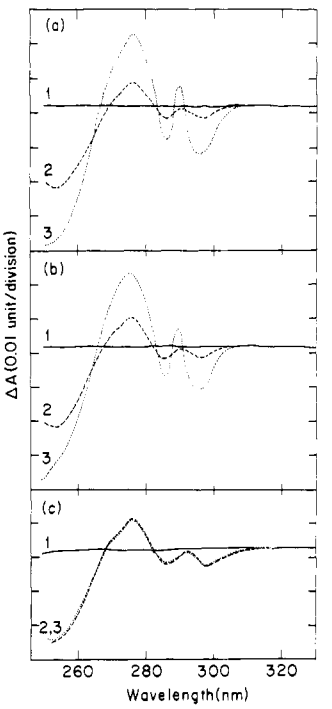

FIGURE 7: Difference spectra measurements on creatine kinase and its S-substituted derivatives. (a) Native enzyme. Into the front compartment of each double-sector cell was pipetted 1.0 mL of a mixture containing 84 mM Tris–acetate buffer, pH 8.5, and 0.028 mM enzyme. Into the rear compartment was pipetted 1.0 mL of a mixture containing 59 mM creatine, 0.18 mM ADP, 3.6 mM magnesium acetate, and 18 mM Tris–acetate buffer, pH 8.5. (1, —) Base line; (2, ---) difference spectrum after mixing the test cell; (3, ···) difference spectrum after addition of 20 μL of 1.0 M $NaNO_3$ to each compartment and remixing the contents of the test cell. (b) *S,S'*-di-CN-CK. The experiment was carried out as described above, except that the front compartment of each cell contained 0.028 mM 75%-active *S,S'*-di-CN-CK. (c) *S*-CN-*S'*-TNB-CK. The experiment was carried out as described above, except that the front compartment of each cell contained 0.028 mM inactive *S*-CN-*S'*-TNB-CK.

Discussion

NTCB modification of creatine kinase involves S-cyanylation and S-arylthiolation in roughly equal proportions.[4] Consider the possibility that these two reactions occur at random, each subunit reacting independently with equal probability of undergoing either cyanylation or arylthiolation. It can be calculated that, statistically, half of the molecules in the dimeric enzyme population will thus be converted into the monocyanomono-TNB derivative, one-quarter of the molecules will be converted into the di-TNB form, and one-quarter of the molecules will be found in the dicyano form. This last derivative, the dicyano enzyme, is 75% active. Hence, the minimal residual activity of the NTCB modification product should thus be one-quarter of 75%, i.e., close to 20%. Experimentally, however, the modified enzyme is practically

[3] The 75%-active isomer *S*-TNB-*S'*-CN-CK produced by the rapid half-cyanolysis of *S,S'*-di-TNB-CK was not available for the spectral experiments in its fully active form, owing to its instability. Experiments on several preparations retaining 40–60% of the activity have shown, however, that addition of nitrate to the E·MgADP·creatine complexes invariably resulted in the characteristic amplification of the difference spectra, indicating formation of the quaternary complex in all cases.

[4] The small deviation from the stoichiometric 1:1 ratio between the two modes of reaction of NTCB is probably due to the subtlety of the conformational difference between the two reactive cysteinyl residues.

inactive, possessing less than 0.5% of its original activity. This means that the modification product contains practically no dicyano enzyme, and hence essentially no di-TNB enzyme, but consists predominantly of the monocyano mono-TNB derivative, which is evidently inactive. Hence, cyanylation and arylthiolation by NTCB are not random but subunit-selective reactions, reflecting a differential chemical environment at the two subunits.

Differential reactivity of the subunits is also exhibited by the kinetic biphasicity of the cyanolysis of *S*,*S*′-di-TNB-CK (Figures 3 and 4). Moreover, the reactivation of the enzyme coincides solely with the faster of the two reactions (Figures 3–5). This clearly indicates that the two parallel cyanolysis reactions are related to two different subunits. It is significant that, in contrast to the biphasicity of the cyanolysis of *S*,*S*′-di-TNB-CK, the cyanolysis under the same conditions of the NTCB-modified enzyme is a smooth pseudo-first-order process throughout the reaction (Figure 2). This shows that, unlike the two TNB groups in *S*,*S*′-di-TNB-CK, the single TNB group in the NTCB-modified enzyme is confined to a single type of subunit. Thus, the subunit selectivity of the NTCB reactions is kinetically confirmed by the subsequent cyanolysis.

NTCB modification produces the catalytically inactive derivative *S*-CN-*S*′-TNB-CK (I). In contrast, the half-cyanolysis product of *S*,*S*′-di-TNB-CK, namely, *S*-TNB-*S*′-CN-CK (II), is highly active (75%). Furthermore, its cyanolysis rate constant (0.3 M^{-1} min^{-1}) is 10 times smaller than that of *S*-CN-*S*′-TNB-CK (3.0 M^{-1} min^{-1}) under the same conditions. Hence, these two derivatives of creatine kinase, possessing such different enzymic and chemical properties, cannot be identical. Since both derivatives have the same composition and both are derived by selective chemical modifications of the same unique pair of sequentially identical cysteinyl residues, they must be isomers. The existence of isomerism in the derivatized dimer suggests that in isomer I the cyano group is linked to subunit A and the TNB group to subunit B, whereas in isomer II the cyano group is linked to subunit B and the TNB group to subunit A. This would imply that the subunits of the dimer are inherently and initially nonequivalent. Since initial nonequivalence of covalently identical subunits can only arise from preexistent asymmetry in the dimeric structure, it would appear that the subunits of creatine kinase are associated asymmetrically. That identical subunits can combine in an asymmetric manner, at least in the crystal state, has been demonstrated in the case of yeast hexokinase B (Steitz et al., 1976). It was found that the subunits of this dimeric enzyme are associated heterologously, being related by a rotation and a translation along a molecular screw axis, and are consequently not in identical environments. It was also observed that the heterologous binding of the subunits is associated with some small differences in the tertiary structure of the two subunits. Our results suggest that a similar situation may exist in creatine kinase in solution. A heterologous structure of the dimer would account for the differential chemical environments existing at the two subunits, as reflected in the nonidentical reactions of NTCB with the two thiol groups. Furthermore, a heterologous association of the subunits could also provide a plausible explanation for the finding that the presence of the bulky *S*-TNB group at subunit A has virtually no effect on the specific activity of the dimer beyond a small inhibitory effect caused by the tiny cyano group at subunit B, but when present at subunit B the single TNB block completely inactivates both subunits [see Figures 3–6 and compare the equally high residual activity (75%) possessed by *S*,*S*′-di-CN-CK and *S*-TNB-*S*′-CN-CK with the inactivity of *S*-CN-*S*′-TNB-CK]. If the subunits of CK are associated heterologously, the two cysteinyl residues would differ from each other in their relation to the intersubunit contact region and therefore selective S-substitution at one subunit would be expected to cause different intersubunit effects than those caused by the same S-substituent at the neighboring subunit. On the other hand, if the subunits were bound symmetrically, the two monocyanomono-TNB derivatives would be expected to be indistinguishable from each other.

It might be argued that asymmetry is induced during DTNB modification and that the active *S*-TNB-*S*′-CN-CK produced by subsequent half-cyanolysis may then undergo a slow structural rearrangement to the inactive form of *S*-CN-*S*′-TNB-CK. The *S*-TNB-*S*′-CN-CK is unstable and tends to lose both activity and TNB content on storage. Although the loss of activity might seem suggestive of a rearrangement to the inactive *S*-CN-*S*′-TNB-CK, the loss of TNB is not consistent with such a rearrangement since the *S*-CN-*S*′-TNB-CK is stable under the same conditions.

Since the dicyano enzyme derivative possesses high residual activity, it is clear that the inactivity of the cyanylated subunit in *S*-CN-*S*′-TNB-CK arises from the presence of the TNB blocking group at the adjacent subunit. This points to the existence of intersubunit interaction within the modified dimer. Further information on the nature of this interaction is provided by the results of the ligand-binding experiments (Figure 7). The quaternary complex E·MgADP·NO_3^-·creatine formed by CK is considered to be a transition-state analogue (t.s.a.) in which the planar trigonal nitrate ion mimics the planar PO_3 group that is transferred between MgADP and creatine during the enzyme-catalyzed reaction (Milner-White & Watts, 1971; Reed & Cohn, 1972; Reed et al., 1978). The conformational changes induced in the enzyme by formation of this complex are reflected in the nitrate-induced changes in the difference spectrum of the E·MgADP·creatine complex. As indicated by the difference spectra, the inactive *S*-CN-*S*′-TNB-CK is capable of forming the E·MgADP·creatine complex at both of its subunits but fails to undergo a further conformational response on subsequent addition of nitrate. However, after displacing its single TNB block by the small cyano group, the resulting 75%-active *S*,*S*′-di-CN-CK regains the enzyme's ability to form the t.s.a. complex at each of the subunits. These results indicate that the presence of the TNB blocking group in one of the subunits eliminates the ability of the neighboring cyanylated subunit to undergo the conformational changes associated with t.s.a. formation.

In summary, the results presented herein suggest that the subunits of CK are associated asymmetrically. In addition, the results demonstrate that specific blocking of the reactive cysteinyl residue in one of the subunits with a TNB group eliminates the ability of the other subunit to form a t.s.a. complex and to catalyze the phosphoryl-transfer reaction.

Acknowledgment

We are indebted to Leon Esterman for the sedimentation equilibrium analyses.

References

Adams, M. J., Blundell, T. L., Dodson, E. J., Dodson, G. G., Vijayan, M., Baker, E. N., Harding, M. M., Hodgkin, D. C., Rimer, B., & Sheat, S. (1969) *Nature (London) 224*, 491–495.

Anderson, W. F., & Steitz, T. A. (1975) *J. Mol. Biol. 92*, 279–287.

Anderson, W. F., Fletterick, R. J., & Steitz, T. A. (1974) *J. Mol. Biol. 86*, 261–269.

Atherton, R. S., & Thomson, A. R. (1969) *Biochem. J. 111*, 797–798.
Atherton, R. S., Laws, J. F., Miles, B. J., & Thomson, A. R. (1970) *Biochem. J. 120*, 589–600.
Bayley, P. M., & Thomson, A. R. (1967) *Biochem. J. 104*, 33C–35C.
Birktoft, J. J., & Blow, D. M. (1972) *J. Mol. Biol. 69*, 187–240.
Brown, C. S., & Cunningham, L. W. (1970) *Biochemistry 9*, 3878–3885.
Brown, H. S., & Fletcher, R. S. (1949) *J. Am. Chem. Soc. 71*, 1845–1854.
Dance, N., & Watts, D. C. (1962) *Biochem. J. 84*, 114P–115P.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.
Degani, Y., & Patchornik, A. (1971) *J. Org. Chem. 36*, 2727–2728.
Degani, Y., & Patchornik, A. (1974) *Biochemistry 13*, 1–11.
Degani, Y., Neumann, H., & Patchornik, A. (1970) *J. Am. Chem. Soc. 92*, 6969–6971.
Der Terrossian, E., & Kassab, R. (1976) *Eur. J. Biochem. 70*, 623–628.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.
Focant, B. (1970) *FEBS Lett. 10*, 57–61.
Focant, B., & Watts, D. C. (1973) *Biochem. J. 135*, 265–276.
Frost, A. F., & Pearson, R. G. (1961) *Kinetics and Mechanism*, Chapter 8, Wiley, New York.
Gosselin-Rey, C., & Gerady, C. (1970) *Biochim. Biophys. Acta 221*, 241–254.
Hoggett, J. G., & Kellett, G. L. (1976) *Eur. J. Biochem. 66*, 65–77.
Kuby, S. A., & Noltmann, E. A. (1962) *Enzymes, 2nd Ed. 6*, 515–596.
Kumudavalli, I., Moreland, B. H., & Watts, D. C. (1970) *Biochem. J. 117*, 513–523.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Levitzki, A., & Koshland, D. E., Jr. (1976) *Curr. Top. Cell. Regul. 10*, 1–40.
Mahowald, T. A. (1965) *Biochemistry 4*, 732–740.
Mahowald, T. A., Noltmann, E. A., & Kuby, S. A. (1962) *J. Biol. Chem. 237*, 1535–1548.
Milner-White, E. J., & Watts, D. C. (1971) *Biochem. J. 122*, 727–740.
Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88–118.
Noda, L., Kuby, S. A., & Lardy, H. A. (1954) *J. Biol. Chem. 209*, 203–210.
O'Sullivan, W. J. (1971) *Int. J. Protein Res. 3*, 139–148.
Price, N. C. (1976) *Biochem. J. 159*, 177–180.
Reed, G. H., & Cohn, M. (1972) *J. Biol. Chem. 247*, 3073–3081.
Reed, G. H., Barlow, C. H., & Burns, R. A., Jr. (1978) *J. Biol. Chem. 253*, 4153–4158.
Reisfeld, R. A., & Small, P. A., Jr. (1966) *Science 152*, 1253–1255.
Smith, J. D., Maggio, E. T., & Kenyon, G. L. (1975) *Biochemistry 14*, 766–771.
Stark, G. R. (1977) *Methods Enzymol. 47*, 129–132.
Steitz, T. A., Fletterick, R. J., & Hwang, K. J. (1973) *J. Mol. Biol. 78*, 551–561.
Steitz, T. A., Fletterick, R. J., Anderson, W. F., & Anderson, C. M. (1976) *J. Mol. Biol. 104*, 197–222.
Thomson, A. R., Eveleigh, J. W., & Miles, B. J. (1964) *Nature (London) 203*, 267–269.
Thomson, A. R., Eveleigh, J. W., Laws, J. F., & Miles, B. J. (1968) in *Homologous Enzymes and Biochemical Evolution* (Van Thoai, N., & Roche, J., Eds.) pp 255–277, Gordon and Breach, New York.
Tulinsky, A., Vandlen, R. L., Morinoto, C. N., Mani, N. V., & Wright, L. H. (1973) *Biochemistry 12*, 4185–4192.
Turner, D. C., Maier, V., & Eppenberger, H. M. (1974) *Dev. Biol. 37*, 63–89.
Vanaman, T. C., & Stark, G. R. (1970) *J. Biol. Chem. 245*, 3565–3573.
Watts, D. C. (1973) *Enzymes, 3rd Ed. 8*, 383–455.
Yue, R. H., Palmieri, R. H., Olson, O. E., & Kuby, S. A. (1967) *Biochemistry 6*, 3204–3227.