EXPERIMENTAL DETERMINATION OF ACTIVATION POTENTIALS OF CK-ISOENZYMES IN HUMAN SERUM AND THEIR SIGNIFICANCE

P. S. RAO, R. G. EVANS and H. S. MUELLER
Department of Cardiology
St. Louis University School of Medicine
St. Louis, Missouri 63104

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SUMMARY. The activation/inactivation of creatine kinase isoenzymes is dependent upon the redox potential of the activator employed. From comparison of the different activating effects with various oxidizing-reducing agents of known redox potentials, a method for determining the mid-point potentials, E_m^7 (pH 7.0, 30°C) for activation of CK-isoenzymes has been developed. The E_m^7 values for CK-MM and MB in human serum were respectively, -0.08 V, -0.31 V (± 0.005 SD). The relative stabilities of CK isoenzymes are a function of their E_m^7 for activation.

A number of different enzymes are activated by certain reducing agents like KCN, cysteine, GSH and inactivated by oxidizing agents (K_3 Fe $(CN)_6$, cystine). Under ideal conditions, such inactivation is reversible by the addition of reducing agents. These results have been interpreted on the basis of the oxidation or reduction of certain substituent sulfhydryl groups in the enzyme molecule which are concerned with enzyme activity; it is known that the enzyme is active when the sulfur is in the reduced -SH form, but inactive when it has the oxidized -S-S configuration. In the case of creatine kinase (ATP: creatine phosphokinase, EC 2. 7. 3. 2) isoenzymes, the presence of sulfhydryl groups in the enzyme molecule has been demonstrated (1), and it has also been shown that as the enzyme activity disappears there is a corresponding loss in sulfhydryl groups (2).

No studies have as yet been made of the CK-isoenzyme activation as a function of the redox potential of the agents employed. However, such an investigation could be expected to yield considerable information on the problem of enzyme activation and inactivation. In this paper, such experiments with human CK isoenzymes (MM, MB and BB) and a variety of oxidizing and reducing agents are described. These studies also enabled us to develop a method for the determination of mid-point potentials for 50% activation of individual CK-isoenzymes.

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MATERIALS AND METHODS

Fresh venous blood samples (20 ml) obtained from patients with documented acute myocardial infarction were allowed to clot for 1 hour, separated by centrifugation and used as such for total CK activation experiments (cf. Fig. 1).

Fresh normal human myocardium (2 g. papillary muscle obtained during open heart surgery for isolation of CK-MM and MB) and fresh human brain tissue (1 g. obtained during autopsy 12 hours after death for isolation of CK-BB), were minced with scissors and homogenized in a Virtis 23 homogenizer in 15 ml of a solution containing 10 mM tris buffer, pH 8.0; 0.25M sucrose and 1 mM EGTA. The tissue was homogenized at 4°C with four 15 second bursts at full speed and then centrifuged (13,000 rpm, 30 min., 4°C). The CK-MM and MB isoenzymes were then separated on a DEAE-cellulose column as described earlier (3). For CK-BB, several 10 µl volumes of brain homogenates were electrophoresed individually, the BB band cut and all the strips eluted into 1 ml of 10 mM tris buffer containing 0.2% BSA.

Total CK was determined at 30°C using Calbiochem Superstat Pak Kit (C.V. 1.53%, n = 32) and CK-MM and MB were measured (4) after separation by electrophoresis of 10 µl volumes of serum (300 V, 30 min., 13 ± 2°C). The appropriate MM and MB bands were cut (from a previously visualized sample) and individual isoenzymes eluted by shaking for 10 minutes into Calbiochem Kit (No. 869215) that contains no creatine phosphate. After addition of 1.8×10^{-2} M creatine phosphate (No. 869247), CK-MM and MB were determined at 37°C (MM C.V. 1.57%, MB C.V. 3.41%, n = 12) and the CK values converted to 30°C.

We first studied the activation-inactivation of total CK containing MM and MB in fresh human serum by various oxidizing-reducing agents (cf. Fig. 1) selected on the basis of their decreasing redox potentials and listed in Table 1. All the redox agents employed were of the highest research grade, and were obtained from Aldrich, J. T. Baker, Calbiochem, Mallinckrodt and Sigma Companies. The solutions (1 M) were prepared just prior to their use and their concentrations varied from 0 to 30 mM in serum. Next, utilizing the known redox potentials of

the above agents as an index of the relative reactivating capacity of individual isoenzymes (cf. Fig. 2) we developed a method for determining the reactivation potential (E_m^7) of individual isoenzymes. Ten µl of 1 M redox agent (9.9 mM in serum) was added to 1 ml partly inactivated serum. Inactivation of individual isoenzymes to about 50% of their initial activity was carried out as follows: 1. For total CK and MM, fresh serum without any sulfhydryl redox activators was used because CK enzymatic activity constituted only 17.8% (8-30, n=15) of total activity. 2. For CK-MB, human serum was inactivated by a) storage at ambient temperature for 48 hours to 53% (44-56, n=12) and, b) by addition of 25 mM oxidized glutathione to 50% (30-65, n=12). 3. Isolated CK isoenzymes obtained from human tissue were inactivated as follows: a) MM to 54% of the initial activity (49-59, n=12) by storing for 72 hours at ambient temperature; b) MB to 58% (43-63, n=12) by storing for 12 hours and c) BB to 40% (45-65, n=12) by storing for 6 hours.

RESULTS AND DISCUSSION

Activating and Inactivating Effects of Various Redox Agents on Total CK in Human Serum: The concentration dependence of the activation/inactivation of CK (U/L) in human serum in the range 0-30 mM of several redox agents (cf. Table 1) was studied. The results are presented in Figure 1. The compounds fell into four groups according to their redox potentials, E_o' (pH 7.0 and 30°C). (1) Group 1 compounds comprised oxidizing agents with high redox potentials like potassium permanganate (+0.176 V), sodium thiosulfate (+0.112 V) and ergothionene (-0.060). They diminished the CK-activity and inactivated the serum. The initial CK activity was 200 U/L (no activator added to serum or GSH in the kit). After addition of 2 and 5 mM of potassium permanganate, CK activity decreased to 80 and 10 U/L respectively. Oxidizing agents with higher redox potentials such as potassium thiocyanate (E_o'= +0.43 V) and potassium iodide (+0.23 V) gave similar results. (2) Group 2 compounds comprised mainly reducing agents with E_o' in the range of -0.14 to -0.24 V such as, glutathione, cysteine, thiodiglycollic acid and N-acetyl cysteine. They

Table 1. Redox Potentials of Various Oxidizing-Reducing Agents (6,7)

No.		E_o', V (pH = 7, 30°C)
1.	Potassium permanganate	+0.176 V
2.	Sodium thiosulfate	+0.112
3.	Ascorbic acid	+0.036
4.	Ergothionene	-0.060*
5.	Thiodiglycolic acid	-0.140*
6.	Cysteine	-0.220*
7.	Glutathione	-0.240*
8.	Mercapto acetic acid	-0.300*
9.	AET (2-Aminoethylisothiouronium bromide, HBr)	-
10.	2-Mercapto ethanol	-0.320*
11.	Dithioerythritol	-0.332*
12.	Dithiothreitol	-0.332*

*These values can vary by as much as 10%. The E_o' of AET is not available and is low. $E_o' = E_o - 0.03$ pH

caused an initial rapid activation of CK activity followed by a slow diminution. For example, with GSH, the CK activity rapidly increased from 200 to a maximum of 1200 U/L at 8 mM, remained the same in the range of 8-15 mM and then diminished to 1050 U/L at 30 mM GSH. (3) Group 3 compounds comprise of reducing agents with lower redox potentials than those of Group 2 (-0.29 to -0.31 V) namely, mercaptoacetic acid and probably AET. Maximal CK activities were obtained by addition of 5mM to serum and remained independent of concentration thereafter. (4) Group 4 compounds comprised of reducing agents with the lowest redox potentials (< -0.32 V) like dithiothreitol, dithioerythritol and 2-mercaptoethanol. They rapidly and completely activated CK. The CK values were substantially higher than those obtained with Groups 2 and 3 activators. The difference corresponded to MB activity in serum determined electrophoretically (±4.6%, n=12). The CK activity was independent of concentration of activator in the range 2-30 mM. In the absence of CK-MB in serum, the increase in activity corresponded to that obtained with Groups 2 and 3 activators.

It is evident from Figure 1 that the enzyme loses its activity in strong

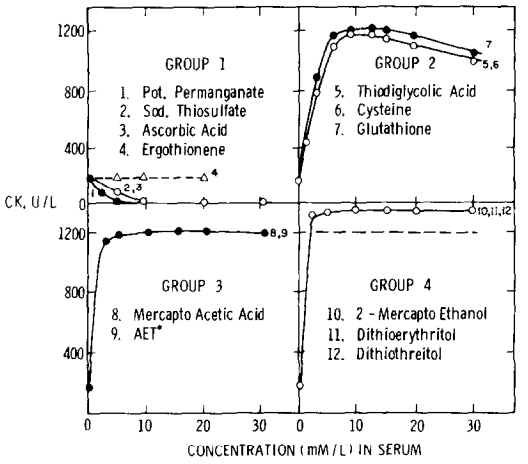

FIGURE 1. Activation-inactivation of total CK in a typical human serum sample (CK-MM 1200 U/L, CK-MB 189). Results are mean of five determinations C.V. 1.4%. *2-Aminoethyl isothiouronium bromide, HBr.

oxidizing solutions. This inactivation is irreversible because of their high redox potentials. This was tested by lowering the E_o' of the system by first adding 10 mM glutathione to serum followed by the oxidizing agent potassium permanganate (5mM). The CK activity increased to 96.8% of its initial value. Activation Potentials of CK-MM and MB: A direct determination of activation/inactivation potentials of CK isoenzymes by electrochemical means is not possible due to difficulties in measuring their actual concentrations and to their interaction with the mercury electrode. A new indirect approach is described here.

It is apparent from Figure 1 that the activation/inactivation of CK-MM and MB in human serum depends on the redox potentials of the various oxidizing-reducing agents. The CK activating ability increased with decreasing redox potential of the agent. Hence, a plot of the CK-reactivation obtained with each redox agent against their E_o' should yield valuable information (cf. Fig. 2). In these experiments, CK-MM and MB in human serum and BB in isolated human tissue were initially inactivated to approximately 50% of their original activities (as described earlier). This initial value prior to addition of 10 mM of redox agents was plotted as 0% reactivation. The

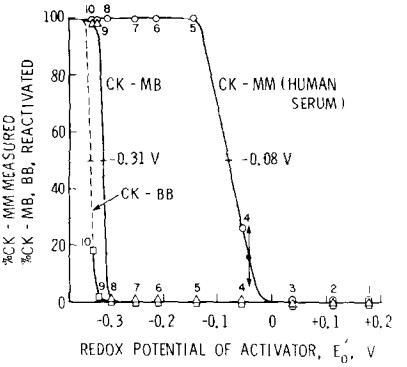

FIGURE 2. Dependence of % reactivation of CK isoenzymes MM, MB and BB upon the redox potential of oxidizing-reducing agents (For CK-MM, C.V. 1.5%, n=12; MB, C.V. 3.4%, n=12; BB, C.V. 4.8%, n=12).

final activity, plotted as 100%, was the original CK-MM or MB value measured with 10 mM dithiothreitol in serum and 9 mM glutathione in assay medium. While compounds of Group 1 showed no reactivation of CK, (in fact, they diminished activity to zero) those of Group 4 produced maximum reactivation of both CK-MM and MB; BB was reactivated by only 18%. Groups 2 and 3 compounds reactivated MM to maximum values (99.8%, 98-102, n=15), but not MB (2.1%, 0-8, n=15). Thus, a titration type of curve was obtained, the middle point of this curve (5) represents 50% reactivation of CK isoenzymes. The corresponding potential is ascribed as the midpoint potential for activation of CK, designated by E_m^7 at pH 7.0 and 30°C. This potential is dependent on the pH, temperature and concentration of activator.

The E_m^7 values obtained from the titration curve, were -0.08 ± 0.005 V for MM and -0.31 ± 0.005 V for MB. CK-MM could be completely reactivated by activators with $E_o' < -0.13$ V, CK-MB by activators with $E_o' < -0.32$ V. The E_m^7 value for CK-BB could not be determined as none of the available activators have low enough redox potentials. Dithiothreitol ($E_o' = 0.33$ V) reactivated CK-BB by only 18%. Therefore, the E_m^7 for CK-BB is below -0.33 V.

It appears from this study that CK-MM is relatively stable over a wide range of E_o' (-0.08 to -0.33 V), with a high E_m^7 and can be easily kept in a

reduced or biologically active state. On the other hand, CK-MB has a low E_m^7 and is rather unstable with a narrow range of E_o' (-0.34 to -0.31 V) and could readily be bound and deactivated in human serum. CK-BB with a very low E_m^7 is deactivated by most of the constituents in serum or air oxidation. These results explain the relative and differing stabilities of the CK isoenzymes in human serum. The similarity of E_o' reactivation curve to a typical pH activity curve is striking and suggests that E_o' as well as pH control may play an important role in enzyme studies. The differences in activation produced by different reductants appears to be related to differences in oxidation-reduction potentials rather than to specific differences in activities of different reductant enzyme complexes.

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