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Binding of ADP to rat liver cytosolic proteins and its influence on the ratio of free ATP/free ADP

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In a cytosolic extract from rat liver, the number and the concentration of ADP-binding sites as well as their dissociation constants were determined by using the rate-of-dialysis technique. Interfering cytosolic adenylate kinase was extracted from the cytosol by affinity chromatography on Ap_5A–agarose, and remaining traces of enzyme activity were inhibited with (+)-catechin. Binding of ADP to cytosolic proteins was increased by poly(ethylene glycol) and decreased by EDTA. The effect of 0.1 mM-EDTA could be reversed by addition of equimolar concentrations of Mn^{2+} or Mg^{2+}. In presence of 5% poly(ethylene glycol), added to increase local protein concentration, two binding sites for ADP were observed, with K_D values of 1.9 μM (site I) and 10.8 μM (site II). The concentration of these binding sites, when extrapolated to cellular protein concentrations, were 30 μM (site I) and 114 μM (site II). It is concluded that a minimum of about 50% of total cytosolic ADP is bound to proteins, and that the ratio of free ATP/free ADP is at least twice that of total ATP/total ADP.

INTRODUCTION

In cell-free systems derived from rat liver, gluconeogenesis is strongly stimulated by increasing ratios of ATP/ADP (Mörikofer-Zwez *et al.*, 1981, 1982; Stoecklin *et al.*, 1986). A linear relationship between the gluconeogenic rate and the ratio of ATP/ADP, ranging from 20 to 60, was observed. Physiological rates of hexose 6-phosphate formation from different substrates were obtained at an ATP/ADP ratio of at least 20. However, cytosolic ratios of ATP/ADP reported for rat liver and rat hepatocytes are not higher than 10 (Siess & Wieland, 1976; Tischler *et al.*, 1977; Akerboom *et al.*, 1978; Soboll *et al.*, 1978). This apparent discrepancy may stem from the fact that in cell-free, highly diluted, systems the adenine nucleotides are essentially present as free compounds (Stoecklin *et al.*, 1986), whereas in the cell they may be bound in part to cytosolic proteins. Since metabolic regulation by adenine nucleotides depends on their free concentration, it seems essential to determine this parameter for cellular conditions.

Several attempts have been made to assess binding of adenine nucleotides to cytosolic proteins and to calculate the free concentration of ATP and ADP in liver. By using a metabolite indicator method, the concentration of free cytosolic ADP in freeze-clamped rat liver has been calculated to be 50 μM (Veech *et al.*, 1979), suggesting that more than 80% of the total cytosolic ADP (0.3 mM; Akerboom *et al.*, 1978) is bound to proteins. This calculation has, however, been questioned by Groen *et al.* (1982), who reported the metabolite-indicator method not to be valid under the conditions employed.

In permeabilized rat liver cells, binding of adenine nucleotides has been studied by Gankema *et al.* (1983). About 30% of the cytosolic ADP was found to be bound at physiological concentrations of ADP, whereas ATP was not bound at all. However, as stated by those authors, the technique employed cannot distinguish between the ADP bound to cytosolic proteins and that tightly bound to cellular membranes.

Several studies using n.m.r. spectroscopy (Cohen, 1983; Iles *et al.*, 1985; Cunningham *et al.*, 1986) have shown that the concentration of n.m.r.-visible free ADP in whole rat liver is not higher than 0.2 mM, corresponding to about 13% of total liver ADP (760 nmol/g liver wet wt.; Faupel *et al.*, 1972). These investigations with whole liver, however, give no answer as to the binding of ADP to cytosolic proteins nor to the number of binding sites and to the binding constants involved. We have therefore attempted to measure binding of ADP in isolated rat liver cytosol, using a rapid-dialysis technique. With this method we have determined binding constants for two ADP-binding sites and have calculated the minimal concentration of ADP-binding sites in the cytosol of hepatocytes. The resulting concentration of free cytosolic ADP is compared with values obtained with other methods. Furthermore, binding of ADP is shown to be influenced by bivalent cations.

MATERIALS AND METHODS

Materials

Enzymes, coenzymes, adenine nucleotides and GSH were purchased from Sigma (St. Louis, MO, U.S.A.). Dithiothreitol and P^1P^5-di(adenosine-5′-)pentaphosphate (Ap_5A) were obtained from Boehringer (Mannheim, Germany). Imidazole, poly(ethylene glycol) 6000, NaCl (suprapur) and KCl (suprapur) were from Merck (Darmstadt, Germany). (+)-Catechin was from Carl Roth K.G. (Karlsruhe, Germany). Agarose–hexane–Ap_5A (type 4) was obtained from Pharmacia (Uppsala, Sweden), Chelex 100 from Bio-Rad (Richmond, CA, U.S.A) and [2-^{3}H]ADP from Amersham International (Amersham, Bucks., U.K.). All other reagents were of the highest purity commercially obtainable.

Abbreviation used: Ap_5A, P^1P^5-di(adenosine-5′-)pentaphosphate.

All solutions were prepared with double-quartz-distilled water. Those used during preparation of the cytosols, chromatography and dialysis were passed through Chelex 100 before use to remove contaminating bivalent cations, except for solutions of EDTA, EGTA, $MgCl_2$ and $MnCl_2$. The glassware used was soaked in 2 mM-EDTA, pH 9.0, for at least 30 min and was then thoroughly washed first with deionized water and then with quartz-distilled water to remove all EDTA.

[2-^{3}H]ADP was freed of contaminating bivalent cations by passage through Chelex 100: 0.5 ml of [2-^{3}H]ADP (100 μCi) in 50% (v/v) ethanol was applied to a column of Chelex 100 (0.5 cm × 4 cm) saturated with the solvent and subsequently eluted with 50% ethanol. The eluate was collected in fractions and the distribution of radioactive material was determined. [2-^{3}H]ADP was eluted in one peak. The peak fractions were combined and the ADP content was determined luminometrically after enzymic conversion into ATP using an ATP bioluminescence CLS kit (Boehringer Mannheim). Specific radioactivity of [2-^{3}H]ADP determined after chromatography on Chelex was 4.4 μCi/nmol. Recovery of radioactivity after chromatography was over 95%.

Radiochemical purity of [2-^{3}H]ADP was tested, on the one hand, by t.l.c. on cellulose F (Merck) with a solvent mixture of butan-1-ol/acetone/acetic acid/5% NH_3/water (9:3:2:2:4, by vol.) and on the other hand by h.p.l.c. on a RP-18 column (250 mm × 4.6 mm; Knauer KG, Berlin, Germany) with a pre-column (40 mm × 4.6 mm), by using a gradient of 0–80% 0.1 M-sodium phosphate buffer (pH 5.5) + 25% (v/v) methanol in 0.1 M-sodium phosphate buffer, pH 5.5. Radiochemical purity of [2-^{3}H]ADP was found by both methods to be over 90%, with no change during chromatography. The preparation contained no other radioactive adenine nucleotides. Before use, the solvent (50% ethanol) was evaporated at room temperature under N_2 and [2-^{3}H]ADP was dissolved in dialysis buffer (see below).

Fed male Wistar rats obtained from Madörin (Frenkendorf, Switzerland) and weighing 200–240 g were used throughout.

Preparation of cytosols

Liver homogenates were prepared as described previously (Mörikofer-Zwez & Walter, 1979), except that homogenization was carried out with a hand-operated loosely fitting spherical glass pestle. The homogenate was centrifuged at 105 000 *g* for 1 h, and 3 ml of the resulting supernatant was gel-filtered through a Sephadex G-25 column (1.0 cm × 20 cm) saturated with 10 mM-imidazole buffer, pH 7.0, containing 1 mM-dithiothreitol (standard buffer). The protein fraction of the cytosol was eluted with the same buffer, and the protein concentration before and after passage through Sephadex was measured in order to determine the dilution factor.

Removal of adenylate kinase from cytosol

Adenylate kinase was extracted from the cytosol by affinity chromatography on Ap_5A–agarose as suggested by Feldhaus *et al.* (1975). Sephadex-filtered cytosol (2 ml) was applied to the column (0.5 cm × 4.0 cm), equilibrated with standard buffer. Proteins were eluted stepwise (*a*) with standard buffer (fraction 1, 5 ml), (*b*) with standard buffer containing 0.5 mM-Ap_5A (fraction 2, 5 ml), (*c*) with standard buffer (fraction 3, 5 ml) and (*d*) with standard buffer containing 200 mM-NaCl (fraction 4, 2.7 ml; fraction 5, 2.3 ml). For elution profiles see Table 1. Fractions 1 and 4 were combined, and 6.8 ml was concentrated 15–30-fold by using Centricon 10 microconcentrators (Amicon Corp., Danvers, MA, U.S.A.). No measurable protein was lost during the concentration procedure. The concentrated protein solutions were combined and diluted to 700 μl with standard buffer. The protein solution was further diluted with an equal volume of standard buffer containing 25 mM-KCl, 20 mM-(+)-catechin and 10% poly(ethylene glycol), except where indicated otherwise [final concns: KCl 12.5 mM, (+)-catechin 10 mM, poly(ethylene glycol) 5%, NaCl (from chromatography) approx. 12–25 mM]. The resulting protein solution, containing 8–11 mg of protein/ml, was used for dialysis.

Determination of ADP binding by the rate-of-dialysis method (Colowick & Womack, 1969)

The dialysis chamber used in the present study was built in accordance with the specifications given by Feldmann (1978). Continuous buffer flow (0.5 ml/min) through the lower chamber was established with a LKB-Microperpex pump (LKB, Bromma, Sweden). The upper and lower dialysis chambers were separated by a Spectrapor 1 dialysis membrane (molecular-mass cut-off 6000–8000 Da; Spectrum Medical Industries, Los Angeles, CA, U.S.A.), soaked overnight in dialysis buffer (standard buffer containing 25 mM-KCl).

For the binding measurements, 0.24 ml of the concentrated solution of cytosolic proteins (see above) was placed in the upper dialysis chamber. Depending on the type of experiment, either 0.01 ml of dialysis buffer or 0.01 ml of an effector solution in dialysis buffer was added to the protein solution. Flow of dialysis buffer through the lower chamber was initiated, and the thermostatically regulated system was allowed to equilibrate at 25 °C for 3 min. Dialysis was started by addition of 0.11 nmol of [2-^{3}H]ADP (0.5 μCi) in 5 μl of dialysis buffer to the upper chamber, together with an appropriate amount of unlabelled ADP in 5 μl of dialysis buffer (under standard conditions 0.22–0.28 nmol, resulting in a final concentration of [2-^{3}H]ADP + ADP of 1.3–1.5 μM). Dialysis buffer passing through the lower chamber was collected in ten fractions of 12 drops each (0.48 ml) directly into scintillation vials. Bound ADP was then set free by addition of 5 μl of 50 mM-ADP to the upper chamber (final concn. 1 mM), and ten further fractions were collected. To each fraction 10 ml of Instagel (Packard Instrument International S.A., Zurich, Switzerland) were added. Radioactivity was determined with a Kontron MR300 liquid-scintillation counter (Kontron AG, Zurich, Switzerland). The rate of dialysis measured in the presence of 1 mM-ADP was taken to correspond to 100% free [2-^{3}H]ADP, since the rate at 0.1 mM- and 1 mM-ADP differed by less than 1%. As the rate of dialysis is proportional to the concentration of free [2-^{3}H]ADP (Colowick & Womack, 1969), the percentage of bound [2-^{3}H]ADP could be calculated as follows:

$$100 - \left(\frac{\text{c.p.m. (fraction 10)}}{\text{mean of c.p.m. (fractions 17–20)}}\right) \times 100$$

The molar concentration was then calculated by multiplying the percentage of bound (or free) [2-^{3}H]ADP

by the concentration of initially added [2-^{3}H]ADP. Examples of dialysis profiles are given in Fig. 1.

The stability of [2-^{3}H]ADP under dialysis conditions was tested in analogous incubation experiments. Reactions were terminated as described previously (Mörikofer-Zwez *et al.*, 1981). 2-^{3}H-labelled adenine nucleotides were analysed by h.p.l.c. as described above for the determination of the radiochemical purity of [2-^{3}H]ADP.

Various determinations

Protein concentrations in the cytosols and in the chromatographic fractions were determined by the method of Lowry *et al.* (1951), with defatted bovine serum albumin as standard. Since (+)-catechin interfered with the protein measurements, the concentration of protein during dialysis was calculated from the protein concentrations in the combined chromatographic fractions 1+4, taking into account all concentration and dilution factors.

Enzyme distribution during chromatography was determined by the following methods: hexokinase (Bergmeyer, 1974), phosphofructokinase (Castano *et al.*, 1979), pyruvate kinase (Bergmeyer, 1974). Fructose-1,6-bisphosphatase was measured at 30 °C with the following assay mixture: 33 mM-Tris/HCl, pH 7.4, 0.67 mM-GSH, 5 mM-$MgCl_2$, 1 mM-EGTA, 0.5 mM-$NADP^+$, 10 units of phosphoglucose isomerase, 3 units of glucose-6-phosphate dehydrogenase and 50 μl of sample in a total volume of 3 ml. The reaction was started by addition of 0.1 mM-fructose 1,6-bisphosphate. Adenylate kinase was measured at 25 °C in the direction of ADP formation with the following assay mixture: 70 mM-triethanolamine buffer, pH 7.5, 120 mM-KCl, 1.2 mM-$MgCl_2$, 1.2 mM-ATP, 0.8 mM-AMP, 0.36 mM-phosphoenolpyruvate, 0.18 mM-NADH, 25 units of pyruvate kinase and 50 units of lactate dehydrogenase in a total volume of 3 ml. The reaction was started by addition of adenylate kinase. ADP was measured as described by Mörikofer-Zwez *et al.* (1981).

RESULTS

Minimization of interfering adenylate kinase activity

One of the main problems when measuring the binding of ADP to cytosolic proteins from rat liver is the conversion of ADP into ATP+AMP by adenylate kinase. The activity of this enzyme is low in liver cytosol *in situ* (Criss, 1970), but high in the intermembrane space of rat liver mitochondria (Criss, 1970). The mitochondrial enzyme may in part be released during tissue homogenization, thereby increasing adenylate kinase activity in isolated liver cytosol. Inhibition of adenylate kinase by EDTA (Gankema *et al.*, 1983) was not feasible, since, as shown below, EDTA itself influenced the binding of ADP. Furthermore, Ap_5A, a potent inhibitor of muscle adenylate kinase, could not be used because it is a relatively poor inhibitor of liver adenylate kinase (Feldhaus *et al.*, 1975) and because, as a structural analogue of the adenine nucleotides, it could interfere with the determination of ADP binding. Therefore, other methods were devised to minimize adenylase kinase activity in the cytosol.

First, rat liver was homogenized with a hand-operated loosely fitting spherical glass pestle. Adenylate kinase activity in the 105000 *g* supernatants of these homogenates was 49±2% (mean±S.E.M., $n = 4$) lower than in supernatants of liver homogenates prepared with a motor-driven standard Teflon pestle, indicating a smaller release of adenylate kinase from damaged mitochondria.

Furthermore, supernatants prepared in this way were gel-filtered through Sephadex G-25 to remove endogenous adenine nucleotides and were then chromatographed on Ap_5A–agarose (see the Materials and methods section). This affinity chromatography resulted in five fractions (Table 1). Fraction 1 contained 60% of the cytosolic proteins, but only 6% of adenylate kinase. Fraction 2, eluted with Ap_5A, contained 16% of the cytosolic proteins. Recovery of adenylate kinase activity in fraction 2 could not be determined correctly, because the enzyme was inhibited during measurements by Ap_5A

Table 1. Chromatography on Ap_5A–agarose: distribution and recovery of protein, adenylate kinase and other cytosolic enzymes

Cytosolic proteins were fractionated on Ap_5A–agarose as described in the Materials and methods section. Results are expressed as means±S.E.M.

Protein or enzyme	Chromatographic fraction...	Cytosolic protein or enzyme in fraction (% of that applied)* 1	2	3	4	5	1+4	Recovery (%)
Protein ($n = 10$)		60±1	16±1	1.9±0.3	19±0.4	1.4±0.2	79±1	98±1
Adenylate kinase ($n = 3$)		6±1	27±5†	1±1	1±1	1±1	8±1	36±4†
Adenylate kinase ($n = 4$), direct elution		8±1	87±2‡					95±2
Pyruvate kinase ($n = 3$)		105±10	2±0	1	3±1	1	108±10	112±9
Phosphofructokinase 1 ($n = 3$)		24±4	72±8	2±0	1±1	1	25±4	99±5
Hexokinase ($n = 2$)		64	6	3	6	4	70	81
Fructose-1,6-bisphosphatase ($n = 2$)		87	2	0	0	0	87	89

* 100% values were: protein 25.0±1.1 mg, adenylate kinase 2.1±0.5 units, pyruvate kinase 5.7±0.3 units, phosphofructokinase 0.7±0.1 unit, hexokinase 1.0 unit and fructose-1,6-bisphosphatase 3.4 units.

† Values are too low, owing to the presence of Ap_5A in the eluate.

‡ Direct elution with 200 mM-NaCl in elution buffer.

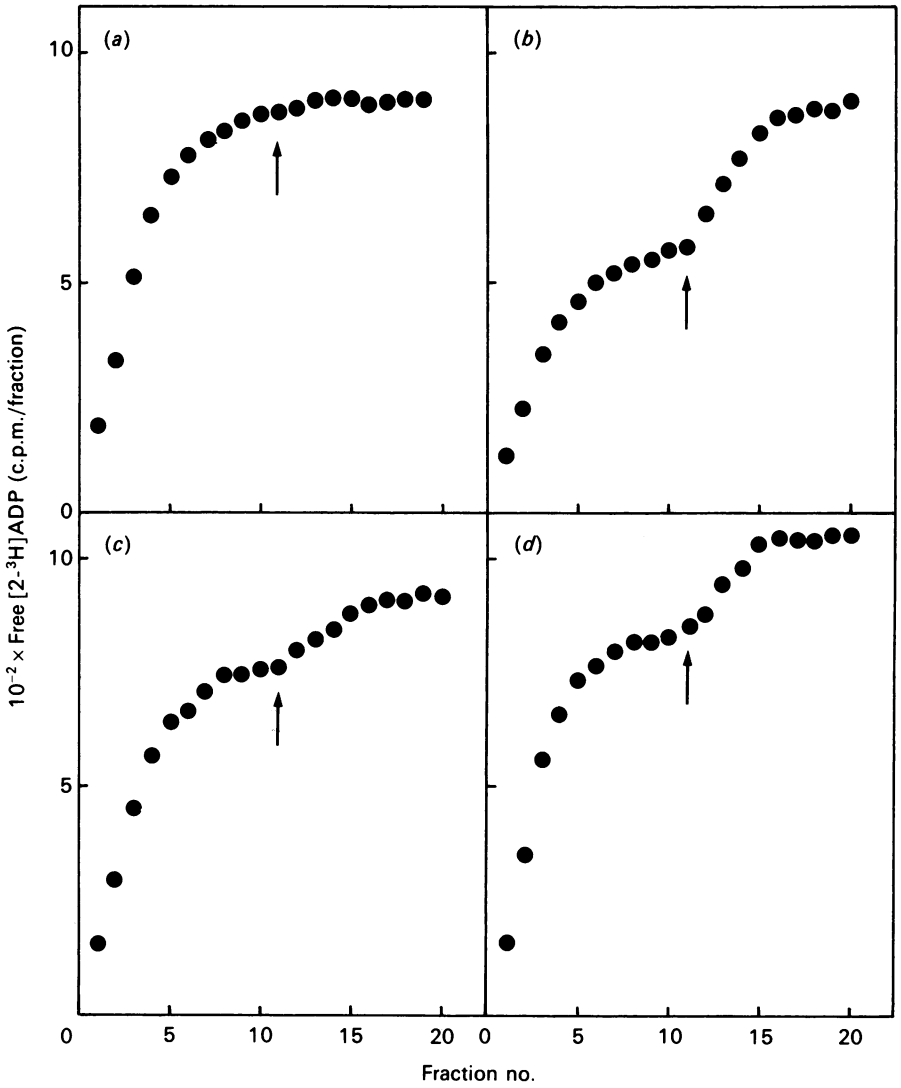

Fig. 1. Typical examples of rate-of-dialysis curves used for the determination of ADP binding

Binding of [2-^{3}H]ADP to cytosolic proteins was measured in the presence of 10 mM-(+)-catechin and 5% poly(ethylene glycol) at different concentrations of protein and [2-^{3}H]ADP as described in the Materials and methods section: (*a*) no protein, [2-^{3}H]ADP 1.5 μM; (*b*) protein 11.6 mg/ml, [2-^{3}H]ADP 1.5 μM; (*c*) protein 3.0 mg/ml, [2-^{3}H]ADP 1.5 μM; (*d*) protein 9.0 mg/ml, [2-^{3}H]ADP 13.7 μM. Arrows indicate addition of unlabelled ADP (final concn. 1 mM).

present in the eluate. Direct elution of adenylate kinase with 200 mM-NaCl in the elution buffer, however, revealed that most of the adenylate kinase activity was retained by Ap_5A–agarose and could be recovered almost quantitatively. A further fraction relatively rich in protein (fraction 4) was eluted after the Ap_5A step with 200 mM-NaCl. Fractions 1 and 4 were combined, concentrated and used for binding measurements as described in the Materials and methods section, whereas fraction 2 was discarded.

The above fractionation procedure implies the risk that other adenine-nucleotide-binding proteins may be lost with the discarded adenylate kinase-containing fraction 2. To assess this risk, the chromatographic distribution of four different enzymes (pyruvate kinase, phosphofructokinase, hexokinase and fructose-1,6-bisphosphatase) which can be expected to have adenine-nucleotide-binding sites was tested. Except for phosphofructokinase 1, the main portion of these enzymes was eluted with fraction 1 (Table 1). Thus adenine-nucleotide-binding proteins are not necessarily bound to Ap_5A–agarose and are thereby extracted from the cytosolic fraction (1+4) used for the binding measurements. Nevertheless, it has to be kept in mind that the binding data presented below are minimal values, and that actual binding of ADP to cytosolic proteins in the cell may be even greater.

As shown in Table 1, about 8% of the adenylate kinase initially present in the cytosol remained in the cytosolic fractions 1+4. This remaining activity still interfered with the binding measurements, and was therefore decreased by about 60% by using (+)-catechin, an inhibitor of adenylate kinase (Mörikofer-Zwez & Walter, 1984). Under these conditions, clear two-step binding curves were obtained (see Fig. 1).

Measurements of ADP binding

Binding of ADP to the proteins present in the cytosolic fractions 1+4 was determined by the rate-of-dialysis

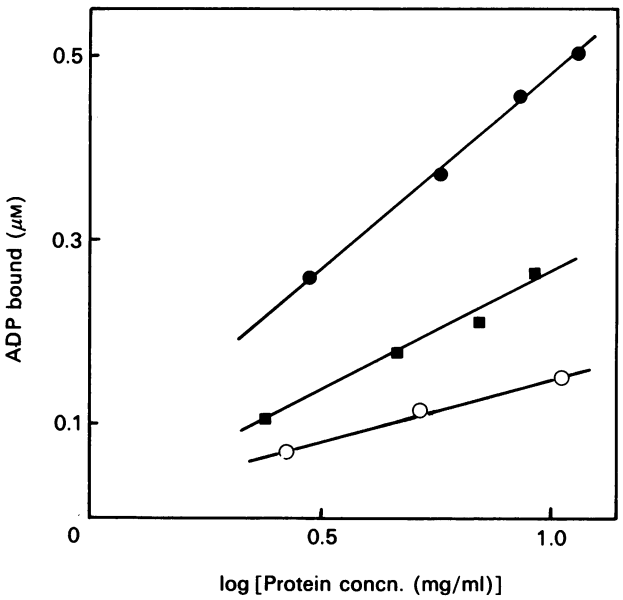

Fig. 2. Relationship between protein concentration and ADP binding

Binding of [2-^{3}H]ADP to cytosolic proteins was measured as described in the Materials and methods section in the presence of 10 mM-(+)-catechin and 5% poly(ethylene glycol) at different concentrations of protein and of [2-^{3}H]ADP in the presence and absence of 1 mM-EDTA: ●, 1.5 μM-[2-^{3}H]ADP, no EDTA; ○, 0.4 μM-[2-^{3}H]ADP, no EDTA; ■, 1.4 μM-[2-^{3}H]ADP, 1 mM-EDTA.

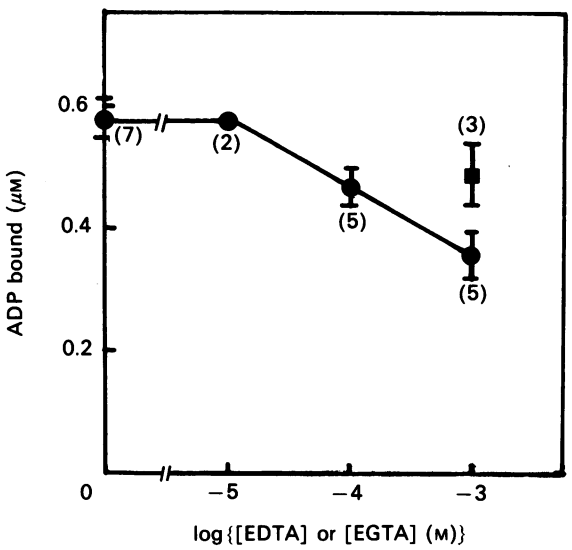

Fig. 3. Effect of EDTA and EGTA on ADP binding

Binding of [2-^{3}H]ADP (1.3–1.5 μM) to cytosolic proteins (10 mg/ml) was measured as described in the Materials and methods section in the presence of 10 mM-(+)-catechin and 5% poly(ethylene glycol) either at different concentrations of EDTA (●) or in presence of 1 mM-EGTA (■). Results are expressed as means ± S.D. for the numbers of independent experiments given in parentheses.

method originally developed by Colowick & Womack (1969) as modified by Feldmann (1978) (for details see the Materials and methods section).

Fig. 1 shows four typical dialysis curves. In Fig. 1(*a*), [2-^{3}H]ADP was added to dialysis buffer containing 10 mM-(+)-catechin and 5% poly(ethylene glycol) in the absence of protein. Here, addition of 1 mM-ADP did not increase the rate of dialysis, showing that ADP binds neither to (+)-catechin nor to poly(ethylene glycol). In the presence of protein (11.6 mg/ml) and [2-^{3}H]ADP (1.5 μM), the latter was in part bound to the protein and was released on addition of 1 mM-ADP (Fig. 1*b*). If the protein concentration was lowered (Fig. 1*c*) or if the initial concentration of [2-^{3}H]ADP was increased (Fig. 1*d*), the percentage of free [2-^{3}H]ADP was increased at the expense of bound [2-^{3}H]ADP. The loss of radioactivity during dialysis was only 1.2 ± 0.1% (mean ± S.D., $n = 8$) during a total of 20 min dialysis time.

Binding of [2-^{3}H]ADP to the cytosolic proteins was linearly related to the logarithm of the protein concentration under all conditions tested (Fig. 2). On the basis of this relationship, all binding measurements were, for the sake of comparison, logarithmically corrected to a protein concentration of 10 mg/ml.

Effects of poly(ethylene glycol) and EDTA on the binding of ADP

Binding of [2-^{3}H]ADP to cytosolic proteins increased on addition of poly(ethylene glycol) to the protein solution. Under standard conditions 0.35 ± 0.05 μM-ADP (mean ± S.D., $n = 3$) was found to be bound to proteins when poly(ethylene glycol) was absent. In the presence of 5% poly(ethylene glycol), bound ADP increased to 0.55 ± 0.04 μM (mean + S.D., $n = 9$). The effect attained at this polymer concentration was maximal, and addition of 10 or 15% poly(ethylene glycol) did not further enhance binding. As the high local protein concentrations in the presence of poly(ethylene glycol) should resemble more closely the situation in the intact cell (Reinhart, 1980; Bosca *et al.*, 1985), all experiments were carried out in the presence of 5% poly(ethylene glycol) except where stated otherwise.

For reasons not known, no definite plateau of free [2-^{3}H]ADP as in Figs. 1(*b*)–1(*d*) was obtained when dialysis was carried out in the presence of added Mg^{2+}, and binding of ADP could therefore not be determined. Parallel incubation experiments (results not shown) suggested that a continuous degradation of [2-^{3}H]ADP to AMP, IMP and inosine in the presence of added Mg^{2+} may be responsible for the phenomenon observed. In the absence of protein, addition of Mg^{2+} did not change the dialysis curve shown in Fig. 1(*a*), excluding an artifactual influence of Mg^{2+} on the dialysis system. The influence of bivalent cations was, however, tested indirectly by addition of EDTA to the protein solution used for dialysis. Under standard conditions (see the Materials and methods section), addition of 1 mM-EDTA decreased bound ADP from 0.35 ± 0.05 μM to 0.20 ± 0.02 μM (mean ± S.D., $n = 3$) in the absence, and from 0.55 ± 0.04 μM to 0.34 ± 0.04 μM (mean ± S.D., $n = 9$ and $n = 6$ respectively) in the presence, of 5% poly(ethylene glycol). Thus binding was decreased by about 40% in both cases. The influence of EDTA was studied in more detail in the experiments shown in Fig. 3. Whereas 10 μM-EDTA had no measurable influence on ADP binding, 0.1 mM- and 1 mM-EDTA decreased binding of ADP by 20 and 38% respectively. However, 1 mM-EGTA decreased binding by only 16%. This rules out Ca^{2+} as the cation involved in ADP-binding, since Ca^{2+} is bound more strongly to

EGTA than to EDTA (Bartfai, 1979). The effect of EDTA could be reversed by Mn^{2+} and by Mg^{2+}: addition of 0.1 mM-EDTA decreased bound ADP from 0.58±0.03 μM ($n = 7$) to 0.47±0.03 μM ($n = 5$), whereas in the presence of 0.1 mM-EDTA + 0.1 μM-Mn^{2+}, bound ADP returned to 0.56±0.06 μM ($n = 3$; results given as mean±S.D.). Using Mg^{2+} instead of Mn^{2+} also brought bound ADP back to 0.56 μM (0.54 and 0.57 μM, $n = 2$). These results indicate that Mn^{2+} and/or Mg^{2+} may be involved in the binding of ADP.

Characterization of binding sites

The relationship between total ADP concentration and bound ADP was determined for total ADP concentrations between 0.43 and 14.8 μM. The Scatchard plot (Fig. 4) derived from these binding measurements clearly reveals the presence of at least two binding sites for ADP. From this plot, dissociation constants and the concentration of ADP-binding sites were calculated from the equation (Colowick & Womack, 1969):

$$B = n - (K_D \times B/F)$$

where B is the concentration of bound and F of free ADP, n is the concentration of binding sites and K_D the dissociation constant. For the first set of binding sites (I) K_D was found to be 1.9 μM and $n = 1.7$ μM, whereas for the second set (II) K_D was 10.8 μM and $n = 6.5$ μM.

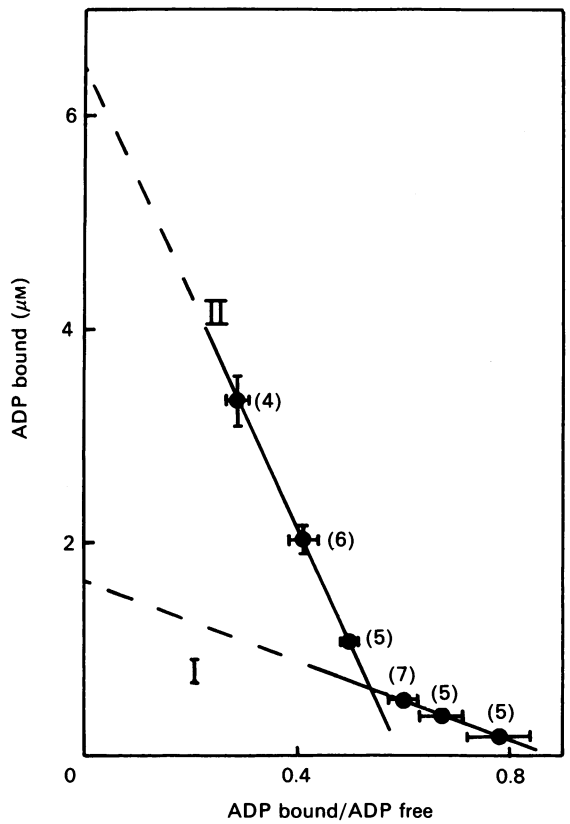

Fig. 4. Scatchard plot for ADP binding

Binding of [2-^{3}H]ADP (0.43–14.8 μM) to cytosolic proteins (10 mg/ml) was measured in the presence of 10 mM-(+)-catechin and 5% poly(ethylene glycol) as described in the Materials and methods section. Results are expressed as means±S.E.M. for the numbers of independent experiments given in parentheses. Where no S.E.M. is given, the value is smaller than the symbols used.

At all concentrations of ADP tested, 1 mM-EDTA decreased binding of ADP by 30–35%, but two binding sites were still observed in the Scatchard plot (not shown). K_D for the first set of binding sites was 2.2 μM and thus similar to the value in the absence of EDTA, whereas n was decreased to 1.0 μM. For the second set of binding sites an accurate determination of K_D and n in presence of EDTA was not possible, owing to the very low binding at higher ADP concentrations. The available data, however, suggest that the concentration of binding sites was also decreased.

From the concentration of binding sites present during dialysis, the concentration of binding sites in the cytosol of intact hepatocytes was extrapolated. Calculations were based on the amount of cytosolic proteins extracted during homogenization of the liver tissue (111±7.7 mg/g of liver; mean±S.D., $n = 13$) corrected for the loss of protein during chromatography on Ap_5A–agarose (see Table 1). The relevant protein concentration in liver-cell cytosol (175.4±12.2 mg/ml) was calculated by using a cellular water content of 0.5 ml/g wet wt. (Brunengraber *et al.*, 1978) that closely corresponds to the values of 0.46 and 0.49 ml/g wet wt. reported in other studies (Soboll *et al.*, 1976; Williamson, 1969). The concentrations of the two ADP-binding sites during dialysis (1.7 μM and 6.5 μM; Fig. 4) were extrapolated to cellular concentrations by using the protein ratio of 175.4/10 (cellular concn./concn. during dialysis). The resulting concentrations of the ADP-binding sites in cytosol were found to be 30 μM for site I, 114 μM for site II and 144 μM for total ADP-binding sites. As mentioned above, these concentrations have to be regarded as minimal values, since it cannot be excluded that some ADP-binding proteins were extracted during the removal of contaminating adenylate kinase.

DISCUSSION

The data in the present paper show that rat liver cytosol contains at least two binding sites for ADP. Dissociation constants of 1.9 μM for site I and 10.8 μM for site II were measured in the absence of chelating agents but in the presence of 5% poly(ethylene glycol). As discussed by Bosca *et al.* (1985), addition of poly(ethylene glycol) as a crowding agent increases local protein concentrations and allows study of enzymes in a physiological state of aggregation. This approach has successfully been used to determine the kinetics of phosphofructokinase (Reinhart, 1980; Bosca *et al.*, 1985) and of pyruvate kinase (Medina *et al.*, 1985), with results similar to those obtained at high, near-physiological, enzyme concentrations. Since the present study attempts to define ADP binding under conditions comparable with those in the cell, most measurements were made in the presence of poly(ethylene glycol), thereby approaching cellular conditions of protein concentration. It was found that poly(ethylene glycol) increased ADP binding by about 60%, indicating a strong influence of protein concentration on the dissociation constant.

Using permeabilized rat liver cells, Gankema *et al.* (1983) observed only one binding site for ADP, with a dissociation constant of 320 μM in the absence and 235 μM in the presence of Mg^{2+}. One of the main differences between the present study and the work of

Gankema *et al.* (1983) is the use of poly(ethylene glycol) to induce high local protein concentrations, instead of permeabilized liver cells. In our system, binding of ADP is 36% lower in the absence of poly(ethylene glycol) than in its presence. Provided that the number of binding sites is not changed, such a decrease implies an increase of the dissociation constant by a factor of about 2. The difference between our results and those of Gankema *et al.* (1983), however, amounts to a factor of at least 20 and therefore cannot be explained by an effect of poly(ethylene glycol). Whether the low-affinity binding site found in permeabilized liver cells is an additional cytosolic binding site lost in the present study during affinity chromatography on Ap_5A–agarose or whether it represents a membrane-associated binding site cannot be decided on the basis of the available data.

The concentrations of both ADP-binding sites in the liver cytosol are considerably higher than the concentration of ADP-binding cytosolic enzymes such as phosphofructokinase 1, phosphoglycerate kinase and pyruvate kinase, which can be calculated to be 0.12 μM, 4.8 μM and 1.3 μM respectively (calculated from data by Sakakibara & Uyeda, 1983; Scrutton & Utter, 1968; Kuntz *et al.*, 1978; Pegoraro & Lee, 1978; Ekman *et al.*, 1976; Seubert & Schoner, 1971). It thus seems unlikely that the observed binding sites for ADP represent binding sites associated with cytosolic enzymes. Furthermore, interference by binding compounds other than proteins during binding measurements seems unlikely, since the cytosols were gel-filtered before use and binding of ADP was not observed in the absence of cytosolic proteins (see Fig. 1). This leads to the conclusion that in the cytosol of hepatocytes there must exist separate soluble ADP-binding proteins of an as-yet undefined nature.

Total n.m.r.-visible free ADP in rat liver *in vivo* has been reported by Cunningham *et al.* (1986) to be 0.2 mM, whereas Cohen (1983) with the same technique found 0.17 mM to be the upper limit for free ADP in perfused rat liver. This n.m.r.-visible free ADP can be assumed to represent essentially free cytosolic ADP, because over 90% of the mitochondrial ADP seems to be bound to proteins (Wilson *et al.*, 1982, 1983). On the other hand, Iles *et al.* (1985) could not detect any n.m.r.-visible ADP in intact tissue, suggesting a concentration of free ADP below 50 μM. A value of about 50 μm for free cytosolic ADP in rat liver was also reported by Veech *et al.* (1979) on the basis of calculations using a combined equilibrium constant for glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase (metabolite-indicator method). As pointed out by Groen *et al.* (1982), this calculated value may, however, be incorrect, because the reaction catalysed by glyceraldehyde-3-phosphate dehydrogenase plus 3-phosphoglycerate kinase is out of equilibrium when flux through the reaction is in the direction of glycolysis, and the use of the metabolite-indicator method leads to erroneous results under these conditions. In the present study a binding capacity of liver cytosol for ADP of 144 μM was found. As the dissociation constants for the two binding sites are in the low-micromolar range, the observed cytosolic ADP-binding sites would lower the concentration of free cytosolic ADP to about 0.16 mM if a total cytosolic ADP concentration of 0.3 mM (Akerboom *et al.*, 1978) is assumed. The value of 0.16 mM for free cytosolic ADP must, however, be regarded as an upper limit, since it cannot be excluded that ADP-binding proteins were lost during the chromatography necessary for the removal of adenylate kinase.

The total concentration of ATP in rat liver cytosol has been reported to be 2.8–3.4 mM (Tischler *et al.*, 1977; Akerboom *et al.*, 1978; Siess *et al.*, 1982). Since little, if any, ATP is bound to cytosolic proteins (Gankema *et al.*, 1983), the concentration of free ATP in rat liver cytosol can be assumed to be about 3 mM. Together with the maximal free cytosolic ADP concentration of 0.16 mM calculated here, a ratio of ATP_{free}/ADP_{free} of at least 19 results. If ADP-binding proteins have been lost owing to the chromatography on Ap_5A–agarose, this ratio would be even higher. The ratio of 19 calculated on the basis of the present results is considerably higher than the cytosolic ratio of ATP_{total}/ADP_{total} of 2.6–10 found by various fractionation techniques (Siess & Wieland, 1976; Tischler *et al.*, 1977; Akerboom *et al.*, 1978; Soboll *et al.*, 1978). However, the present results agree well with previous findings showing that a ratio of at least 20 is a prerequisite for obtaining physiological rates of gluconeogenesis in cell-free systems (Mörikofer-Zwez *et al.*, 1982; Stoecklin *et al.*, 1986).

Binding of ADP to cytosolic proteins was found to depend on the presence of bivalent cations, Mg^{2+} and Mn^{2+} being about equally effective. The relatively high concentration of EDTA needed to obtain a reduction of ADP binding indicates that the bivalent cations are tightly bound. For two reasons it seems furthermore likely that they are bound to proteins and not to ADP. (*a*) The solutions used for dialysis were Chelex-treated (see the Materials and methods section), which should have removed ADP-bound bivalent cations, and the cytosol was freed of low-molecular-mass compounds by gel filtration. (*b*) In order to obtain an effect on ADP binding, the concentration of EDTA had to exceed the concentration of ADP by a factor of about 70 (Fig. 3), but the association constants for MgADP and MnADP (Hammes & Hurst, 1969; Colman, 1972; O'Sullivan & Perrin, 1964) are considerably lower than those for the corresponding metal–EDTA complexes (Bartfai, 1979). Whether changes in the binding of bivalent cations to proteins play a role in the regulation of free cytosolic ADP and thereby influence the activity of adenine-nucleotide-dependent processes in rat liver cytosol is an open question.

Further investigations will be necessary to identify the ADP-binding proteins and to evaluate their potentially regulatory effect on the concentration of free ADP in rat liver cytosol.

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