Phosphorylation of adenosine in anoxic hepatocytes by an exchange reaction catalysed by adenosine kinase

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The elevation of adenosine levels induced by anoxia in isolated rat hepatocytes has been shown to result mainly from an arrest of the recycling of the nucleoside by adenosine kinase [Bontemps, Vincent and Van den Berghe (1993) Biochem. J. **290**, 671–677]. To assess the activity of the latter enzyme in intact hepatocytes, incorporation of radioactive adenosine into the cells' adenine nucleotides was measured. Unexpectedly, despite the near-absence of ATP in anoxic cells, 40 % of 50 μM [8-^{14}C]adenosine was still incorporated into adenylates over 5 min. Moreover, whereas unlabelled and labelled adenosine were utilized in parallel in normoxic cells, uptake of [8-^{14}C]adenosine did not correspond to a net disappearance of adenosine in anoxic cells. Addition of 1 mM unlabelled adenosine to anoxic hepatocytes in which the adenine nucleotides had been prelabelled with [U-^{14}C]adenine induced an immediate loss of their radioactivity. The latter was recovered in the form of adenosine, but the size of the adenylate pool was not modified. Taken together, these results suggest the occurrence of an exchange reaction between AMP and adenosine. Incubation of Sephadex G-25-filtered high-speed supernatants of rat liver with 20 μM [8-^{14}C]adenosine, 10 mM $MgCl_2$ and 1 mM AMP resulted in the labelling of AMP in the total absence of ATP. This labelling was influenced by effectors of both adenosine kinase and cytosolic IMP–GMP 5′-nucleotidase; the latter is known to catalyse an exchange reaction [Worku and Newby (1982) Biochem. J. **205**, 503–510]. Chromatography of cytosolic fractions of rat liver on DEAE-Sepharose, followed by Sephacryl S-200 and AMP–Sepharose, demonstrated that the exchange reaction between adenosine and AMP co-purified with adenosine kinase. It is concluded that incorporation of labelled adenosine into adenine nucleotides should not be considered to be proof of adenosine kinase activity in anoxia.

INTRODUCTION

As reported in the accompanying paper (Bontemps et al., 1993), the concentration of adenosine increases several-fold upon induction of anoxia in suspensions of hepatocytes isolated from fasted rats. Investigation of the mechanism of this elevation showed that it results mainly from an arrest of the rephosphorylation of the nucleoside by adenosine kinase. The latter was demonstrated by the absence of an effect of 5-iodotubercidin (ITu), a specific inhibitor of adenosine kinase (Henderson et al., 1972a), on the concentration of adenosine and on the production of its catabolites. The phosphorylation of adenosine in intact cells can also be assessed by measuring the incorporation of radioactive adenosine into their adenine nucleotides. In the present work we report the unexpected observation that, in anoxic hepatocytes, labelled adenosine can still be incorporated into AMP, despite the near-complete absence of ATP. This incorporation corresponds neither to a net utilization of adenosine nor to a net synthesis of adenine nucleotides. Further studies led to the conclusion that this incorporation results from an exchange reaction between adenosine and AMP, catalysed by adenosine kinase.

Part of this work has been presented at a Symposium (Bontemps et al., 1991).

MATERIALS AND METHODS

Chemicals

[U-^{14}C]Adenine (270 Ci/mol), [8-^{14}C]adenosine (50 Ci/mol), [8-^{14}C]AMP (55 Ci/mol) and [8-^{14}C]IMP (56 Ci/mol) were purchased from Amersham International (Amersham, Bucks., U.K.). DEAE-Sepharose Fast Flow, Sephacryl S-200, 5′-AMP–Sepharose 4B and Sephadex G-25 (fine grade) were from Pharmacia (Uppsala, Sweden). Poly(ethylene glycol) (PEG) 6000 was from UCB (Brussels, Belgium). The sources of all other chemicals have been given previously (Bontemps et al., 1988, 1993). All chemicals were of analytical grade.

Experiments with isolated hepatocytes

Hepatocytes were isolated as previously described (Van den Berghe et al., 1980) from male Wistar rats which were fasted overnight. The cells were resuspended in Krebs–Ringer bicarbonate buffer supplemented with 10 mM glucose and 1 % BSA, and gassed with O_2/CO_2 (19:1). The final concentration of hepatocytes was 50 mg/ml. The cells were preincubated for 15 min before the induction of anoxia, with or without 0.05 μM deoxycoformycin (dCF) as indicated. In some experiments, 1 μM [U-^{14}C]adenine was added 5 min before induction of anoxia to label the adenine nucleotide pool as described previously (Van den Berghe et al., 1980). Anoxia was induced by replacing O_2/CO_2 by N_2/CO_2 (19:1). Experiments shown are representative of at least three studies that gave similar results.

Analytical methods

Concentrations and radioactivities of adenine nucleotides and adenosine were measured in perchloric acid extracts as described before (Van den Berghe et al., 1980). Protein was determined by the method of Bradford (1976), with bovine γ-globulin as the standard.

Abbreviations used: 2,3-BPG, 2,3-bisphosphoglycerate; dCF, deoxycoformycin; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; ITu, 5-iodotubercidin; PEG, poly(ethylene glycol).

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Experiments with cytosolic fractions of rat liver

All procedures were performed at 4 °C. For the preparation of cytosolic fractions, rat liver was homogenized in 2 vol. of 25 mM Hepes, pH 7.1, containing 20 mM KCl and 1 mM dithiothreitol (buffer A). The homogenate was centrifuged for 15 min at 8000 *g*, and the resulting supernatant was further centrifuged for 45 min at 100000 *g*. This high-speed supernatant was dialysed overnight against 100 vol. of buffer A and thereafter filtered on Sephadex G-25 fine to remove all traces of ATP and other low-molecular-mass metabolites. Absence of nucleotides (less than 0.1 μM) in the filtrate was confirmed by h.p.l.c. (Hartwick and Brown, 1975).

DEAE-Sepharose chromatography was performed on high-speed supernatants that were first mixed with PEG at a final concentration of 30 %. The mixture was gently stirred for 30 min at 4 °C, followed by centrifugation for 15 min at 8000 *g*. The pellet was dissolved in 50 ml of buffer A without KCl, and applied on a 1.6 cm × 20 cm DEAE-Sepharose column equilibrated with buffer A containing 10 mM KCl. The column was washed with 150 ml of the same buffer and eluted with a 10–600 mM gradient of KCl in buffer A, at a flow rate of 60 ml/h.

Purification of adenosine kinase

For the purification of adenosine kinase, the buffers used were supplemented with leupeptin and antipaïn (10 μg/ml) in the steps from the preparation of the homogenate up to and including chromatography on Sephacryl S-200.

The fractions eluted from the DEAE-Sepharose column which contained adenosine kinase activity were pooled and concentrated 5–10-fold on Amicon YM 10 filters. A 2 ml portion of the concentrate was applied on a Sephacryl S-200 column (1.6 cm × 58 cm). Elution was accomplished with buffer A containing 0.2 M KCl. Active fractions were pooled and 6 ml of the pool was applied to a 0.4 cm × 10 cm column containing 1.2 ml of 5′-AMP–Sepharose 4B equilibrated with 25 mM Hepes, pH 7.1, and 200 mM KCl, supplemented with dithiothreitol to a final concentration of 5 mM (buffer B). After rinsing with 10 ml of buffer B containing 0.5 M KCl, followed by 5 ml of buffer B without KCl, adenosine kinase was eluted with 1 mM adenosine in buffer B without KCl. The eluate was collected in albumin (1 mg/ml) to stabilize the enzyme. The specific activity of adenosine kinase, measured at 0.1 mM adenosine, was approx. 0.6 μmol/min per mg of protein, which represents an approx. 700-fold purification from the resuspended and Sephadex G-25-filtered 30 % PEG pellet. The enzyme preparation was free of AMP deaminase, 5′-nucleotidase, adenosine deaminase and adenylate kinase. SDS/PAGE, performed according to Laemmli (1970) and using Coomassie Blue as the tracking dye, revealed a single protein band.

Enzyme assays

Adenosine–AMP exchange reaction

This reaction was assayed by measuring the incorporation of [8-^{14}C]adenosine into AMP in the absence of ATP. Incubations were performed at 37 °C in a medium containing 50 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 0.05 μM dCF or 0.1 mM *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) to inhibit adenosine deaminase, 1 mM AMP, 20 μM [8-^{14}C]adenosine and 10 or 50 μl of a Sephadex G-25-filtered high-speed supernatant, as indicated, or 20 μl of a column fraction, in a total volume of 100 μl. The reaction was arrested by spotting 10 μl of the incubation medium on to cellulose t.l.c. plates, on which carrier solutions (50 nmol) of AMP and adenosine had been applied. After migration of adenosine in water, the spot corresponding to AMP, remaining at the origin, was cut out and its radioactivity counted. The exchange activity was calculated from the quantity of radioactive AMP formed.

Adenosine kinase

Adenosine kinase activity was measured in a medium containing 50 mM Hepes, pH 7.2, 5 mM $MgCl_2$, 3 mM ATP, 0.1 mM EHNA, 100 μM [8-^{14}C]adenosine, 0.25 mM phosphoenolpyruvate, 2 units/ml pyruvate kinase, 3.6 units/ml myokinase and an appropriate amount of enzyme in a total volume of 100 μl. The reaction was arrested as described for the adenosine–AMP exchange reaction except that, in addition, a carrier solution of ATP had been applied. Owing to the excess of myokinase and pyruvate kinase, AMP is at least 95 % converted into ATP. The activity of adenosine kinase was calculated from the radioactivity incorporated into ATP, which remained at the origin upon development of the t.l.c. plates in water.

Other enzymes

5′-Nucleotidase activities with 1 mM [8-^{14}C]IMP or [8-^{14}C]AMP as substrates, and the activities of AMP deaminase, adenosine deaminase and adenylate kinase were measured as described previously (Bontemps et al., 1988).

RESULTS

Experiments with isolated hepatocytes

Effect of anoxia on the incorporation of labelled adenosine into adenine nucleotides

To verify that, as concluded in the accompanying paper (Bontemps et al., 1993), adenosine kinase was inactive in anoxic conditions, 50 μM [8-^{14}C]adenosine was added to isolated hepatocyte suspensions after 20 min of incubation in either O_2/CO_2 or N_2/CO_2, in the absence or in the presence of ITu (Figure 1). At 5 min after the addition of [8-^{14}C]adenosine to the normoxic cell suspension, the labelled nucleoside had completely disappeared (Figure 1a): 85 % was incorporated into the adenine nucleotides (Figure 1c), and 15 % was recovered under the form of its deamination products (Figure 1e). However, 5 min after the addition of [8-^{14}C]adenosine under anoxic conditions, 42 % of the labelled nucleoside was still incorporated in the adenine nucleotides (Figure 1d); 35 % of adenosine remained in the cell suspension (Figure 1b) and 25 % was recovered as deamination products (Figure 1f). Thus, although N_2/CO_2 decreased ATP to nearly undetectable levels (see accompanying paper), the incorporation of [8-^{14}C]adenosine into the adenine nucleotides still represented the major pathway of its metabolism in anoxia, reaching about 25 % of the value recorded in the presence of O_2/CO_2. As in normoxia, this residual incorporation was inhibited by ITu (Figures 1c and 1d). Although a precise rate of phosphorylation of adenosine was difficult to calculate owing to dilution of exogenous [8-^{14}C]adenosine by endogenous unlabelled adenosine, it reached up to 140 nmol/min per g in N_2/CO_2. This rate is at least 3-fold higher than the rate of production of adenosine in anoxia (see accompanying paper), and should thus be sufficient to rephosphorylate all adenosine produced under anoxic conditions.

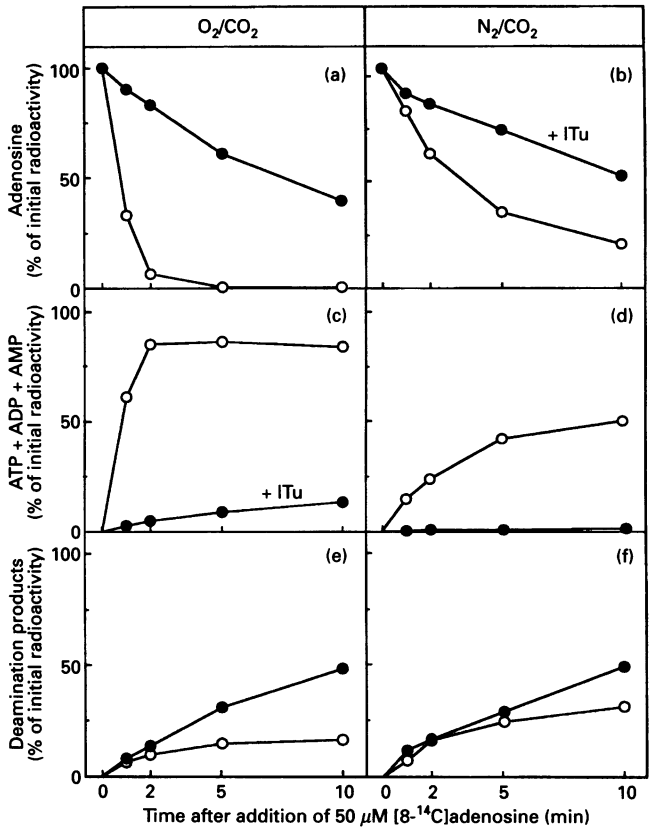

Figure 1 Metabolism of 50 μM [8-^{14}C]adenosine in hepatocyte suspensions incubated in N_2/CO_2 or O_2/CO_2

Isolated hepatocytes were incubated for 20 min in the presence of O_2/CO_2 (left panels) or N_2/CO_2 (right panels), without (○) or with (●) 100 μM ITu, before addition of adenosine.

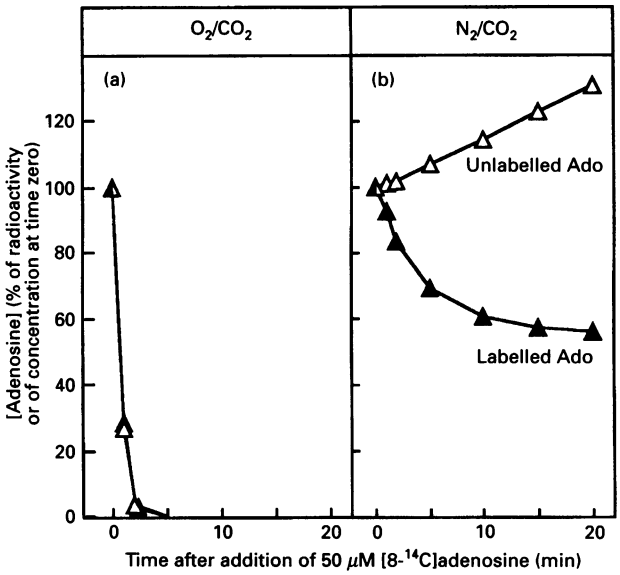

Figure 2 Disappearance of labelled as compared with unlabelled adenosine in the presence of O_2/CO_2 or N_2/CO_2

Hepatocytes, preincubated with 0.05 μM dCF, were incubated for 20 min in the presence of O_2/CO_2 (**a**) or N_2/CO_2 (**b**), before addition of 50 μM [8-^{14}C]adenosine. The radioactivity (▲) and concentration (△) of the nucleoside were determined. At zero time, the total adenosine concentration was 50 μM in O_2/CO_2, but 85 μM in N_2/CO_2, owing to the accumulation of endogenous adenosine in the presence of dCF under anoxic conditions.

When hepatocytes were incubated with 5 mM KCN instead of N_2/CO_2, or with lower concentrations (5 and 0.5 μM) of [8-^{14}C]adenosine, the latter to avoid changing the concentration of endogenous adenosine, results similar to those depicted in Figure 1 were obtained: in all of these experiments, a detectable adenosine kinase activity seemed to remain despite the near-complete absence of ATP (results not shown).

Figure 2 depicts an experiment identical to that described in Figure 1, except that the hepatocytes had been preincubated with dCF to inhibit adenosine deaminase (Henderson et al., 1977). Under these conditions, the uptake of adenosine only reflects adenosine kinase activity. As shown in Figure 2(a), unlabelled and labelled adenosine disappeared strictly in parallel in the presence of O_2/CO_2. In marked contrast, in N_2/CO_2 (Figure 2b), the uptake of labelled adenosine did not correspond to a net disappearance of unlabelled adenosine. It might thus be concluded, notwithstanding the results presented in the accompanying paper (Bontemps et al., 1993), that adenosine could be simultaneously phosphorylated and produced under anoxic conditions.

Influence of the addition of unlabelled adenosine on prelabelled adenine nucleotides

Addition of 1 mM adenosine to normoxic isolated hepatocytes induces an increase in their total adenine nucleotide pool (Lund et al., 1975; Marchand et al., 1979; Bontemps et al., 1983). In hepatocytes which have been preincubated with [U-^{14}C]adenine in order to label their adenine nucleotides, addition of 1 mM adenosine induces immediate formation of [^{14}C]adenosine at the expense of the labelled adenine nucleotides (Bontemps et al., 1983). This is explained by the fact that the high concentration of unlabelled adenosine competes with [^{14}C]adenosine, produced from the ^{14}C-labelled adenine nucleotides, for phosphorylation by adenosine kinase. In the absence of adenosine recycling, we would expect the addition of a high concentration of unlabelled adenosine to neither increase the adenine nucleotide pool nor induce a decrease in its radioactivity. Accordingly, the addition of 1 mM adenosine to anoxic hepatocytes did not modify their adenine nucleotide pool (results not shown); however, as illustrated in Figure 3, it induced an immediate loss of radioactivity from the adenine nucleotide pool (Figure 3a), which was recovered quantitatively in the form of [^{14}C]adenosine (Figure 3b). This phenomenon resulted in a decrease in the specific radioactivity of the adenine nucleotides (to about 50% of its initial value within 20 min) suggesting, once again, that their synthesis and degradation operated simultaneously. This effect of unlabelled adenosine was suppressed by ITu and was not mimicked by inosine (results not shown).

Taken together, the results depicted in Figures 1–3 could be explained, without contradicting the conclusion of the accompanying paper, by the presence of an exchange reaction between AMP and adenosine, resulting in the production of phosphorylated adenosine in the absence of ATP. The results also predict that this exchange reaction should be inhibited by ITu.

Experiments in cell-free systems

Evidence for an exchange reaction between AMP and adenosine in rat liver cytosol

When a Sephadex G-25-filtered high-speed supernatant of rat liver was incubated in the presence of 20 μM [8-^{14}C]adenosine,

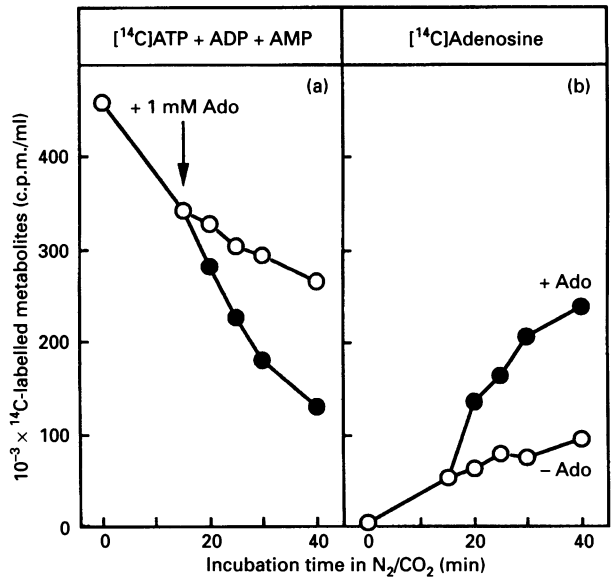

Figure 3 Effect of the addition of unlabelled adenosine on the catabolism of prelabelled adenine nucleotides in anoxic hepatocytes

Hepatocytes were preincubated in the presence of 0.05 μM dCF and 1 μM [U-^{14}C]adenine. Radioactivity in the adenine nucleotides (**a**) and in adenosine (**b**) was determined in the absence (○) and in the presence (●) of adenosine (1 mM), added 15 min after induction of anoxia.

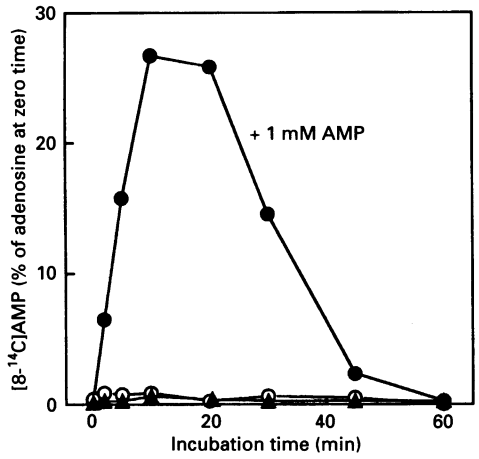

Figure 4 Synthesis of [8-^{14}C]AMP from AMP and [8-^{14}C]adenosine in a gel-filtered supernatant of rat liver

Incubations were performed in the presence of 10 mM $MgCl_2$, 10 mM P_i, 20 μM [8-^{14}C]adenosine and 50 μl of a filtered high-speed supernatant, without other additions (○), with 1 mM AMP (●) or with 1 mM AMP plus 100 μM ITu (▲).

10 mM $MgCl_2$ and 10 mM P_i, no formation of [8-^{14}C]AMP could be detected. However, if 1 mM AMP was added to this incubate, the nucleotide became labelled (Figure 4). This suggested the occurrence of an exchange reaction between free [8-^{14}C]adenosine and the unlabelled adenosine moiety of AMP. The rate of the reaction was difficult to quantify precisely owing to simultaneous degradation of AMP in the crude cytosol preparation, as illustrated in Figure 4 by the transitory accumulation of the labelled nucleotide. Nevertheless, the rate of exchange reached at least 20 nmol/min per g liver wet weight. The exchange

Table 1 Effect of various compounds on the adenosine–AMP exchange reaction

The exchange reaction was measured as described in the Materials and methods section with 20 μM [8-^{14}C]adenosine, 1 mM AMP, 10 mM P_i and 10 μl of a filtered high-speed supernatant. Incubation times were 2, 5 and 10 min. Activities are expressed as a percentage of the radioactivity incorporated from adenosine into AMP in the absence of additions. Results are means of duplicate assays.

Additions	Activity (%)
None	100
ITu (100 μM)	1
5′-Deoxy-5′-aminoadenosine (1 mM)	11
6-Methylmercaptopurine riboside (1 mM)	48
S-Adenosylhomocysteine (1 mM)	75
2,3-BPG (3 mM)	490
5′-Deoxy-5′-isobutylthioadenosine (5 mM)	21
Diadenosine tetraphosphate (1 mM)	32

reaction required $MgCl_2$ (not shown). P_i was not needed, but its addition to crude cytosol preparations reduced the rate of AMP degradation, most likely owing to its inhibitory effect on cytosolic 5′-nucleotidase (Van den Berghe et al., 1977).

In accordance with our prediction, incorporation of [8-^{14}C]-adenosine into AMP was inhibited by ITu (Figure 4 and Table 1) and also by other inhibitors of adenosine kinase, namely 5′-deoxy-5′-aminoadenosine (Miller et al., 1979), *S*-adenosylhomocysteine and 6-methylmercaptopurine riboside (Palella et al., 1980). Inosine, deoxyadenosine and AICA (*N*-1-ribosyl-5-aminoimidazole-4-carboxamide) riboside, at a concentration of 1 mM, did not influence the reaction (results not shown). On the other hand, the exchange reaction was stimulated by 2,3-bisphosphoglycerate (2,3-BPG), a specific stimulator of the cytosolic IMP–GMP 5′-nucleotidase (Bontemps et al., 1988, 1989). The latter result led us to investigate the influence of other effectors of this cytosolic 5′-nucleotidase, which has been shown to catalyse a nucleoside exchange (Worku and Newby, 1982; Keller et al., 1985; Johnson and Fridland, 1989). It was found that the exchange reaction was inhibited by 5′-deoxy-5′-isobutylthioadenosine, which inhibits cytosolic 5′-nucleotidase (Skladanowski et al., 1989), but also by diadenosine tetraphosphate, known to be the most potent stimulator of this enzyme (Pinto et al., 1986). It was verified that the various effectors listed in Table 1 did not inhibit or stimulate the degradation of AMP into adenosine in the crude extracts, so as to mimic stimulation or inhibition respectively of the exchange reaction. To determine whether the exchange reaction between adenosine and AMP was effected by adenosine kinase or by cytosolic 5′-nucleotidase, crude liver extracts were subjected to further purification.

DEAE-Sepharose chromatography of rat liver cytosol

Cytosolic fractions prepared from rat liver as described in the Materials and methods section were chromatographed on DEAE-Sepharose. Figure 5(a) shows that the adenosine–AMP exchange activity was eluted with the washing buffer, before the start of the linear gradient of KCl. As already observed in the crude high-speed supernatant, the exchange reaction activity eluted from the DEAE-Sepharose column was strikingly stimulated by 2,3-BPG; furthermore, it was dependent on the presence of AMP in the assay, and was suppressed by ITu (results not shown). A first peak of IMP 5′-nucleotidase activity was eluted at 80 mM KCl, and a second at 340 mM KCl (Figure 5b). The

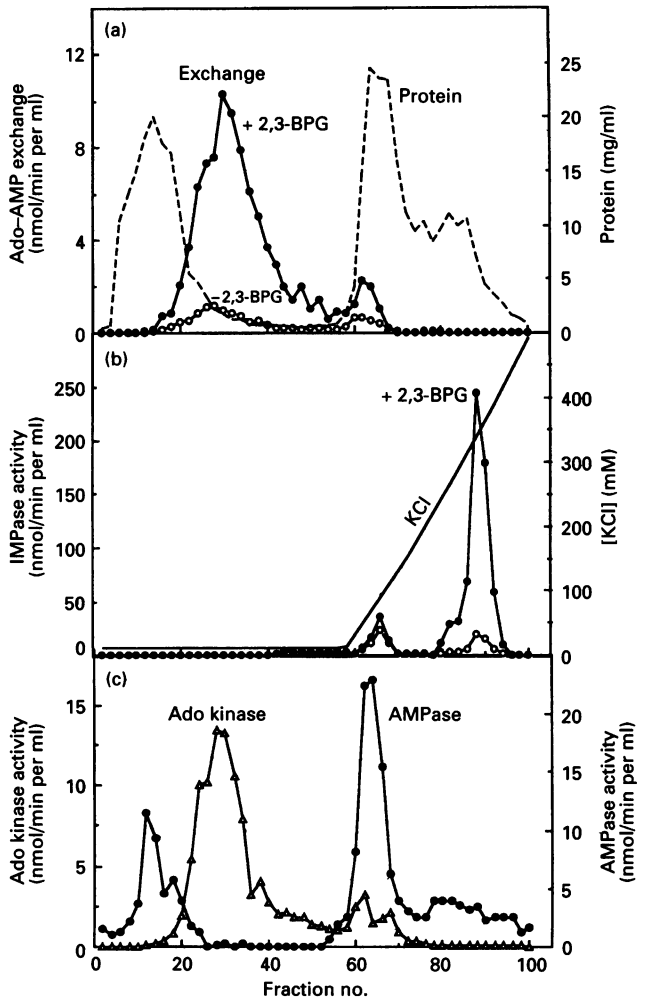

Figure 5 DEAE-Sepharose chromatography of rat liver cytosol

The column, prepared as described in the Materials and methods section, was first washed with 150 ml of a buffer containing 25 mM Hepes, pH 7.1, 1 mM dithiothreitol and 10 mM KCl, and then eluted with a linear gradient of KCl, shown in (**b**). Fraction volume was 5 ml during washing, and 2 ml after the start of the gradient, at fraction no. 42. Adenosine–AMP exchange (**a**) and IMP 5′-nucleotidase activity (**b**) were measured without (○) and with (●) 3 mM 2,3-BPG.

activity of the second peak was strongly stimulated by 2,3-BPG, which indicates that it corresponds to the activity of the cytosolic IMP–GMP 5′-nucleotidase (Bontemps et al., 1988, 1989). These results clearly show that this enzyme is not responsible for the adenosine–AMP exchange reaction. Figure 5(c) shows the elution profile of the adenosine kinase and AMP 5′-nucleotidase activities: adenosine kinase activity, eluted with the washing buffer, coincides with the elution profile of the adenosine–AMP exchange activity.

Co-purification of the adenosine kinase and adenosine–AMP exchange activities

The most active fractions eluted from the DEAE-Sepharose, containing both the adenosine kinase and adenosine–AMP exchange activities, were concentrated and gel-filtered on Sephacryl S-200, as described in the Materials and methods section.

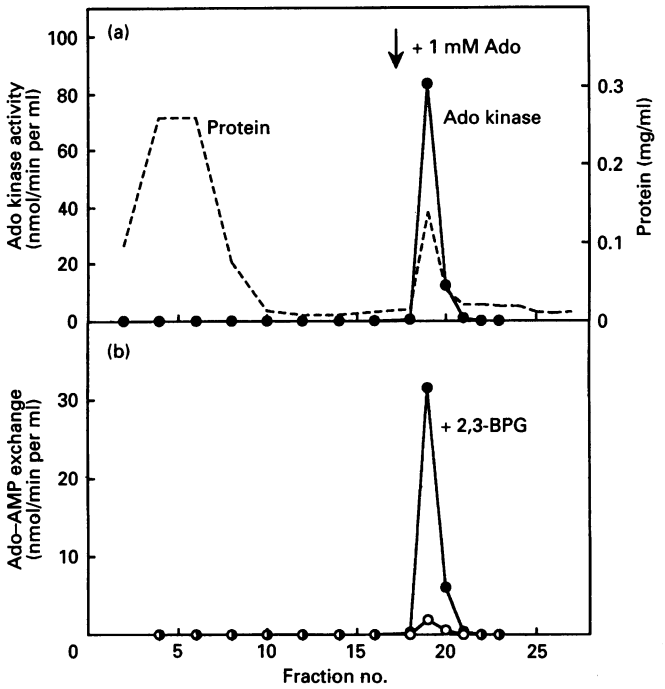

Figure 6 Elution profiles of the adenosine kinase (a) and adenosine–AMP exchange (b) activities from 5′-AMP–Sepharose 4B

Pooled fractions (6 ml) from the Sephacryl S-200 column were applied and eluted as described in detail in the Materials and methods section. Buffer B containing 0.5 M KCl was applied at fraction 6, buffer B without KCl at fraction 14, and buffer B containing 1 mM adenosine at fraction 18. Fraction volume was 1.2 ml. The exchange activity (**b**) was measured without (○) and with (●) 3 mM 2,3-BPG.

It was observed that the adenosine–AMP exchange activity co-eluted again with that of adenosine kinase, after the bulk of proteins (results not shown). Further purification of adenosine kinase was carried on by affinity chromatography on 5′-AMP–Sepharose 4B, usually a key step in this procedure (Andres and Fox, 1979; Yamada et al., 1980; Fisher and Newsholme, 1984). Figure 6 shows that both activities were adsorbed on the resin, whereas most of the proteins were not. Elution with 1 mM adenosine released both the adenosine kinase and adenosine–AMP exchange activities in the same fractions. As also illustrated in Figures 5 and 6, the ability of 2,3-BPG to stimulate the adenosine–AMP exchange reaction was maintained during all steps of the purification. In the absence of 2,3-BPG, the exchange activity represents only 3–5% of the adenosine kinase activity, but it reaches nearly 50% in the presence of 2,3-BPG. We have verified that 2,3-BPG acted as a stimulator and not as a phosphate donor: no exchange reaction was observed in the presence of 2,3-BPG when AMP was absent from the incubation mixture. The effect of 2,3-BPG on the activity of adenosine kinase was also investigated: a stimulatory effect of only about 20% was observed. Preliminary studies have shown that the various effectors of the exchange reaction in the crude extract, listed in Table 1, had the same effect on the purified enzyme preparation.

DISCUSSION

This study shows that in anoxic hepatocytes, in which it was concluded that adenosine kinase is inactive owing to depletion of ATP (Bontemps et al., 1993), adenosine can still be phosphorylated by an exchange reaction catalysed by adenosine kinase.

The possibility that an exchange reaction between adenosine and AMP was taking place was indicated by the observation that hepatocytes depleted in ATP were still able to incorporate radioactive adenosine into AMP (Figure 1). However, this incorporation of labelled adenosine into adenine nucleotides of anoxic hepatocytes did not correspond to a net utilization of adenosine (Figure 2b), demonstrating that no net phosphorylation of adenosine occurred in anoxia. The observation that addition of 1 mM unlabelled adenosine did not modify the size of the adenine nucleotide pool, but induced a transfer of radioactivity from prelabelled adenine nucleotides to adenosine, corroborates the hypothesis of an exchange reaction.

Experiments using cytosolic fractions of rat liver, in which great care was taken to remove all ATP, confirmed that labelled AMP could be formed from radioactive adenosine and unlabelled AMP in the absence of ATP. The fact that this reaction was inhibited by ITu, a powerful inhibitor of adenosine kinase (Henderson et al., 1972a), and by other inhibitors of the enzyme such as 5′-deoxy-5′-aminoadenosine (Miller et al., 1979), 6-methylmercaptopurine riboside and *S*-adenosylhomocysteine (Palella et al., 1980), and by diadenosine tetraphosphate (Rotllan and Miras Portugal, 1985), suggested that the exchange reaction was catalysed by adenosine kinase. The observation that the adenosine–AMP exchange activity co-purified with adenosine kinase in various protein chromatography systems such as DEAE-Sepharose, Sephacryl S-200 and AMP–Sepharose, corroborated this hypothesis.

IMP–GMP cytosolic 5′-nucleotidase has been shown to catalyse a nucleoside exchange between inosine and IMP, but not between adenosine and AMP (Worku and Newby, 1982). It has also been reported to phosphorylate pharmacological nucleoside analogues, namely acyclovir (Keller et al., 1985) and dideoxyinosine (Johnson and Fridland, 1989). The latter authors have shown, moreover, that this phosphorylation is stimulated by two potent stimulators of IMP-GMP 5′-nucleotidase, i.e. diadenosine tetraphosphate (Pinto et al., 1986) and 2,3-BPG (Bontemps et al., 1988, 1989). Although 2,3-BPG was also found to potently stimulate the adenosine–AMP exchange reaction, in contrast with diadenosine tetraphosphate which was inhibitory, the cytosolic IMP–GMP 5′-nucleotidase was clearly separated from the adenosine–AMP exchange reaction.

One major conclusion to be drawn from the discovery of the adenosine–AMP exchange reaction is that the incorporation of labelled adenosine into adenine nucleotides should not always be considered to correspond to a net synthesis of adenine nucleotides. Although the adenosine–AMP exchange activity represents only a few per cent of the adenosine kinase activity under basal conditions, the fact that it can be stimulated by 2,3-BPG to represent half of the adenosine kinase activity suggests that the exchange reaction may be not negligible *in vivo*, and may perhaps be regulated, not by 2,3-BPG, the concentration of which is only about 0.05 μM in the liver (Tauler et al., 1987), but by other phosphoric esters which remain to be identified. The physiological role, if any, of the adenosine–AMP exchange reaction remains to be determined.

Several studies of the reaction catalysed by adenosine kinase have led to the conclusion that it proceeds by an ordered sequential mechanism in which ATP (Henderson et al., 1972b) or adenosine (Palella et al., 1980; Rotllan and Miras Portugal, 1985; Hawkins and Bagnara, 1987) is the first substrate to bind, and AMP the last product to be released. This reaction sequence is, however, not compatible with an adenosine–AMP exchange reaction, which should involve a phosphorylated enzyme intermediate. Nevertheless, a two-site Ping-Pong mechanism with a phosphorylated enzyme intermediate has been proposed for adenosine kinase from murine leukaemia L1210 cells (Chang et al., 1983). Whereas these authors succeeded in demonstrating phosphorylation of the enzyme by ATP, they could not isolate a phosphorylated enzyme after incubation with AMP. Further studies with purified adenosine kinase from rat liver are necessary to verify this point.

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REFERENCES

Andres, C. M. and Fox, I. H. (1979) J. Biol. Chem. **254**, 11388–11393
Bontemps, F., Van den Berghe, G. and Hers, H. G. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 2829–2833
Bontemps, F., Van den Berghe, G. and Hers, H. G. (1988) Biochem. J. **250**, 687–696
Bontemps, F., Vincent, M. F., Van den Bergh, F., van Waeg, G. and Van den Berghe, G. (1989) Biochim. Biophys. Acta **997**, 131–134
Bontemps, F., Mimouni, M. and Van den Berghe, G. (1991) Adv. Exp. Med. Biol. **309A**, 317–320
Bontemps, F., Vincent, M. F. and Van den Berghe, G. (1993) **290**, 671–677
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Chang, C. H., Cha, S., Brockman, R. W. and Bennett, L. L., Jr. (1983) Biochemistry **22**, 600–611
Fisher, M. N. and Newsholme, E. A. (1984) Biochem. J. **221**, 521–528
Hartwick, R. A. and Brown, P. R. (1975) J. Chromatogr. **112**, 651–662
Hawkins, C. F. and Bagnara, A. S. (1987) Biochemistry **26**, 1982–1987
Henderson, J. F., Paterson, A. R. P., Caldwell, I. C., Paul, B., Chan, M. C. and Lau, K. F. (1972a) Cancer Chemother. Rep. Part 2 **3**, 71–85
Henderson, J. F., Mikoshiba, A., Chu, S. Y. and Caldwell, I. C. (1972b) J. Biol. Chem. **247**, 1972–1975
Henderson, J. F., Brox, L., Zombor, G., Hunting, D. and Lomax, C. A. (1977) Biochem. Pharmacol. **26**, 1967–1972
Johnson, M. A. and Fridland, A. (1989) Mol. Pharmacol. **36**, 291–295
Keller, P. M., McKee, S. A. and Fyfe, J. A. (1985) J. Biol. Chem. **260**, 8664–8667
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lund, P., Cornell, N. W. and Krebs, H. A. (1975) Biochem. J. **152**, 593–599
Marchand, J. C. Lavoinne, A., Giroz, M. and Matray, F. (1979) Biochimie **61**, 1273–1282
Miller, R. L., Adamczyk, D. L., Miller, W. H., Koszalka, G. W., Rideout, J. L., Beacham, L. M., III, Chao, E. Y., Haggerty, J. J., Krenitsky, T. A. and Elion, G. B. (1979) J. Biol. Chem. **254**, 2346–2352
Palella, T. D., Andres, C. M. and Fox, I. H. (1980) J. Biol. Chem. **255**, 5264–5269
Pinto, R. M., Canales, J., Günther Sillero, M. A. and Sillero, A. (1986) Biochem. Biophys. Res. Commun. **138**, 261–267
Rotllan, P. and Miras Portugal, M. T. (1985) Eur. J. Biochem. **151**, 365–371
Skladanowski, A. C., Sala, G. B. and Newby, A. C. (1989) Biochem. J. **262**, 203–208
Tauler, A., Gil, J., Bartrons, R. and Carreras, J. (1987) Comp. Biochem. Physiol. **86B**, 11–13
Van den Berghe, G., van Pottelsberghe, C. and Hers, H. G. (1977) Biochem. J. **162**, 611–616
Van den Berghe, G., Bontemps, F. and Hers, H. G. (1980) Biochem. J. **88**, 913–920
Worku, Y. and Newby, A. C. (1982) Biochem. J. **205**, 503–510
Yamada, Y., Goto, H. and Ogasawara, N. (1980) Biochim. Biophys. Acta **616**, 199–207

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